Recognition and cytokine signalling pathways in host defence against *Aspergillus fumigatus*

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Colofon

The research presented in this thesis was performed at the Department of Internal Medicine in the Radboud University Medical Center, The Netherlands

Cover/back illustrations

Cover: Sketch of the *Aspergillus fumigatus* conidiophore surrounded by immune cells
Back: From top to bottom sketch of the germination of *Aspergillus fumigatus* conidia into germlings, short hyphae and hyphae.

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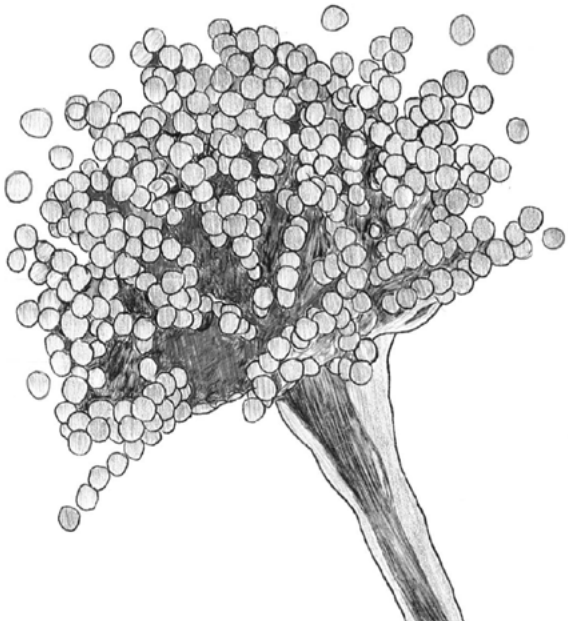
Chapter 1

General introduction and outline of the thesis



Introduction

Aspergillus is a saprophytic fungus that plays an important role in carbon recycling from dead organic materials, therefore it can be found virtually everywhere. The fact that *Aspergillus* species are able to grow in such a wide variety of conditions and can utilize a wide range of organic molecules explains its success in being ubiquitously present in the environment. During the growth of *Aspergillus*, spores (also called conidia) are produced in conidiophores (see cover illustration of each chapter). In 1792, the Italian biologist Pier Antonio Micheli discovered that these conidiophores resemble the form of an aspergillum which is used to disperse holy water by priests, hence the name *Aspergillus* [1]. Disturbance of the conidiophores allows them to effectively and abundantly disperse spores in the environment. This abundance is reflected by the fact that the spores of *Aspergillus spp.* are amongst the dominant fungal components found during air sampling [2,3], which includes the air in hospitals (especially in old buildings and during renovations [3-5]). This results in the fact that humans, and more importantly hospitalized patients, are continuously exposed by inhalation of *Aspergillus* spores.

The *Aspergillus* genus consists of over 870 species [6], which are predominantly saprophytes. Yet, *Aspergillus fumigatus* distinguishes itself together with *A. favus, A. niger, A. tereus, A. clavatus* and *A. nidulans* from the other *Aspergillus* species by being capable of causing infections and allergic disease in humans. Among the various *Aspergillus* species, *A. fumigatus* is the most common opportunistic pathogen and allergen [7].

Aspergillus as an opportunistic pathogen

Hundreds of *Aspergillus* conidia enter the human body daily via the airways and, due to their small size of 2-3 μm [8], easily reach the alveoli in the lung. The cilia on epithelial cells in the airways and the innate immune system, such as alveolar macrophages, are capable of efficiently removing inhaled conidia from the airways. Thereby preventing the conidia from germinating into hyphae, without inducing an inflammatory reaction or pathology. Since *Aspergillus* is so efficiently cleared, it rarely causes infections or induces inflammation. Interestingly, a high burden of *Aspergillus* conidia in the environment could lead to abnormal high exposure and even colonization of *Aspergillus* in the lungs [9]. Despite the fact that *Aspergillus* rarely causes infections within the general population there are several predisposing factors, primarily related to the immune status of the host, that can make individuals susceptible to opportunistic invasive infections and even allergic reactions [8], often collectively called aspergillosis (Figure 1). These diseases primarily manifest within the lungs due to inhalation of conidia, but *Aspergillus* is also known to cause infections of the sinuses, brain, wounds and eyes. The latter occurs primarily in trauma injuries, which are often associated with agriculture, due to fungal spores entering the cornea [10].

Before the Second World War, aspergillosis was considered to be of little importance since it only rarely occurred [11]. However, advances in medical care have resulted in an increasing population of immunocompromised patients that are at risk to develop opportunistic infections with *A. fumigatus* [12]. Especially patients who have a compromised immune status due to haematological malignancies, bone marrow transplantation (hematopoietic stem cell transplants) [13-15] or solid

organ transplantation [16-18], are at risk to develop lethal pneumonia and invasive aspergillosis (IA). The development of aspergillosis in these patients is primarily caused by their therapy, which suppresses the first line of the antifungal host defence in the lungs [14,15,19], that is required for clearing inhaled conidia from the lungs. Chemotherapy has a massive immunosuppressive side effect by being cytotoxic to fast proliferating immune cells leading to neutropenia. The myeloablative therapy before stem cell or organ transplantation results even in a more complete suppression of the immune system. Furthermore, additional risk factors are neutralization of immune effector functions by treatment with high dose corticosteroids, old age, chronic diseases like diabetes and development of graft versus host disease (GVHD) [15].

Besides medically induced immunosuppression, congenital immunodeficiencies can also lead to a immunocompromised status of the host and an increased susceptibility to IA. A rare genetic defect in the NADPH oxidase complex leads to a disease called chronic granulomatous disease (CGD), which is characterized by an inability of immune cells to induce reactive oxygen species (ROS), which specifically predisposes these patients to develop invasive infection with *A. fumigatus* and *A. nidulans* [19,20].

On top of acute invasive disease in immunocompromised individuals, *Aspergillus* can also cause chronic pulmonary aspergillosis (CPA) in patients that do not necessarily have a known severely immunocompromised status. CPA was already described by John Hughes Bennet in 1842 [21]. Pre-existing lung damage and pulmonary diseases, such as sarcoidosis, tuberculosis and chronic obstructive pulmonary disease (COPD) are primary risk factors for the development of CPA [22,23]; primarily because damaged pulmonary tissue facilitates saprophytic fungal growth. Especially,

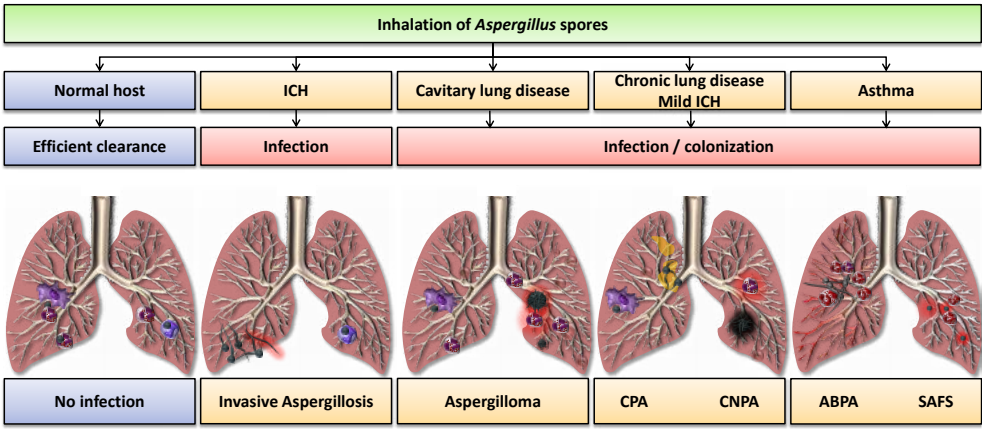


Figure 1| Schematic overview of the infections caused by *A. fumigatus*
(adapted from Soubani *et al* 2002 Chest). In the normal host efficient clearance leads to removal of conidia and no progression to infection, whereas in immunocompromised hosts (ICH) *Aspergillus* spores will not be cleared and invasive aspergillosis (IA) can develop. Patients with cavitary lung disease such as tuberculosis and sarcoidosis or chronic lung disease and mildly immunocompromised hosts can become infected and colonized by *Aspergillus* which can lead to the formation of an aspergilloma or chronic pulmonary aspergillosis. Individuals with asthma can present with severe asthmatic fungal sensitization (SAFS) but can also develop an infection characterized by hypersensitive allergic responses called allergic bronchopulmonary aspergillosis (ABPA).

in cavitary lung diseases necrotic tissue in the lung allows a niche for saprophytic fungal growth. Moreover, *Aspergillus* can grow into a "fungus ball", called an aspergilloma, filling the pre-existing cavities in the lung [22,24,25]. In this disease, also called chronic cavitary pulmonary aspergillosis (CCPA), the growth of an aspergilloma is often limited by the physiological barriers, but in some cases it can gradually expand and destroy surrounding host tissues.

Due to the complicated status of patients who develop IA and CPA, the mortality as a result of aspergillosis in patients is 50%, despite improving treatment possibilities [26]. Even a higher mortality rate is reported in patients infected with resistant strains, in mis-diagnosed cases and when diagnosis delayed. Recent estimates suggest that there are yearly over 200.000 life threatening infections with *A. fumigatus* with a mortality rate between 30 and 90% [26].

In contrast to these invasive forms of aspergillosis, found in a host with a compromised immune status or pre-existing lung damage, fungal particles and also *A. fumigatus* itself can cause the development of pulmonary allergic responses in an individual with a hypersensitive immune status [27-30]. Asthma with severe asthmatic fungal sensitization, also known as SAFS [28], is characterised by positive *Aspergillus*-antigen skin tests [27,29] and high serum levels of *Aspergillus*-specific immunoglobulin E [29,31]. Asthmatic patients and patients with cystic fibrosis (CF) are mainly effected, and the lungs of these patients can become colonized with *Aspergillus* leading to a continuous exposure to the fungus. The hyperreactive T-helper (Th)2 response in these patient groups, can progress to detrimental inflammatory pathologies also termed allergic bronchopulmonary aspergillosis (ABPA). ABPA was first described in asthmatic patients [30,32], but was later also associated with CF [30,33]. Worldwide, ABPA is expected to affect around 4 million asthmatic and cystic fibrosis patients [26,34].

Host defence against *Aspergillus*

Individuals that are able to induce an effective innate antifungal host response will not develop invasive, chronic or allergic aspergillosis, therefore the reports of aspergillosis in immunocompetent patients are very rare. The first step in this protective immunity is the clearance of inhaled spores from the lungs. To efficiently clear conidia from the lungs, they need to be recognised by the hosts immune system but also by the pulmonary epithelium that is also able to recruits immune cells. The immune system employs various pattern recognition receptors (PRRs) that recognise distinct pathogen associated molecular patterns (PAMPs) that are present on and in the fungal cell wall but not in the host itself. The relevance of an effective pathogen recognition is highlighted by the observation that *Drosophila* fruitflies deficient for the pathogen recognition molecule Toll succumb to lethal *Aspergillus* infections [35]. Of interest, this observation led to the discovery of the Toll-like receptors (TLRs), which now have been implicated in inducing host responses against bacterial, viral, parasitic and fungal pathogens. In contrast to the *Drosophila* immune recognition, evolution led to redundant system cosisting of numerous in PRRs and immune signalling pathways in the human immune system. To date 13 different TLRs are known in mammals of which TLR1 to 10 are expressed in humans [36]. The progressing research on immune recognition of fungi like *Aspergillus*

also led to the discovery of another class of pattern recognition receptors, namely the C-type lectin receptors (CLRs) [37]. CLRs are similar to TLRs, capable of recognising specific moieties on the fungal cell wall, and thereby activate immune cells and the subsequent antifungal host response. Another class of PRRs are the NACHT-LRR receptors (NLRs), to which the Nucleotide Oligomerization Domain (NOD)-receptors belong. Although these receptors have been described to primarily play a role in the recognition of bacterial peptidoglycan derivates, there is increasing evidence that they play a role in inducing host responses against *A. fumigatus* [38-40].

Downstream of the PRRs several signalling cascades of various kinases and adaptor molecules will eventually activate several mechanisms. The immune cell can be triggered to phagocytose the pathogen and transcription factors activate the immune cell by switching on transcription of inflammatory genes, such as those encoding cytokines. Cytokines regulate a large variety of processes within the immune system. Chemotactic cytokines (chemokines) released by infected epithelial cells or activated innate immune cells regulate the recruitment of additional immune cells to the site of the infection, other cytokines regulate immune cell activation and proliferation, while some of them also exert metabolic effects.

Responses by antigen specific T-helper responses also play a significant role in the host response against *Aspergillus*. These T-helper responses serve to amplify and strengthen the innate immune responses. The Th17 response, that plays a significant role in host defence against extracellular bacteria, fungi, parasites and viruses [41], is characterised by expression of the transcription factor RORγt and production of the cytokines interleukin(IL)-17 and IL-22. IL-17 facilitates neutrophil recruitment by inducing chemokine release in bronchial epithelial cells [42], whereas IL-22 promotes the antimicrobial defence of the epithelium by promoting release of anti microbial peptides (AMPs) [43]. The Th1 response is characterized by interferon(IFN)γ expression and plays a prominent role in fungal clearance [44,45] and protection against *Aspergillus*-induced detrimental Th2 responses [46]. The Th2 response regulates allergic inflammation and humoral (antibody-mediated) adaptive immune responses. However, in the host defence against aspergillosis this response is primarily associated with development of allergic immune responses that contribute to the severity of the infection due to immunopathology [29,46].

Due to the redundancy of the human immune system, individuals that are deficient in one of the immune systems components that are involved in the host defence against *Aspergillus* do not immediately develop invasive *Aspergillus* infections like the toll deficient *Drosophila* flies. However, in a patient with a severely compromised innate immune system, relatively small deficiencies in the receptors and cytokine signalling pathways, required for anti-*Aspergillus* host defence, could result in an increased susceptibility to aspergillosis. Knowledge on the pattern recognition and cytokine pathways that orchestrate the induction of the antifungal host defence against aspergillosis is therefore crucial for the design of genetic studies that can be used to predict susceptibility to aspergillosis, and for the development of targeted and personalized immunotherapy.

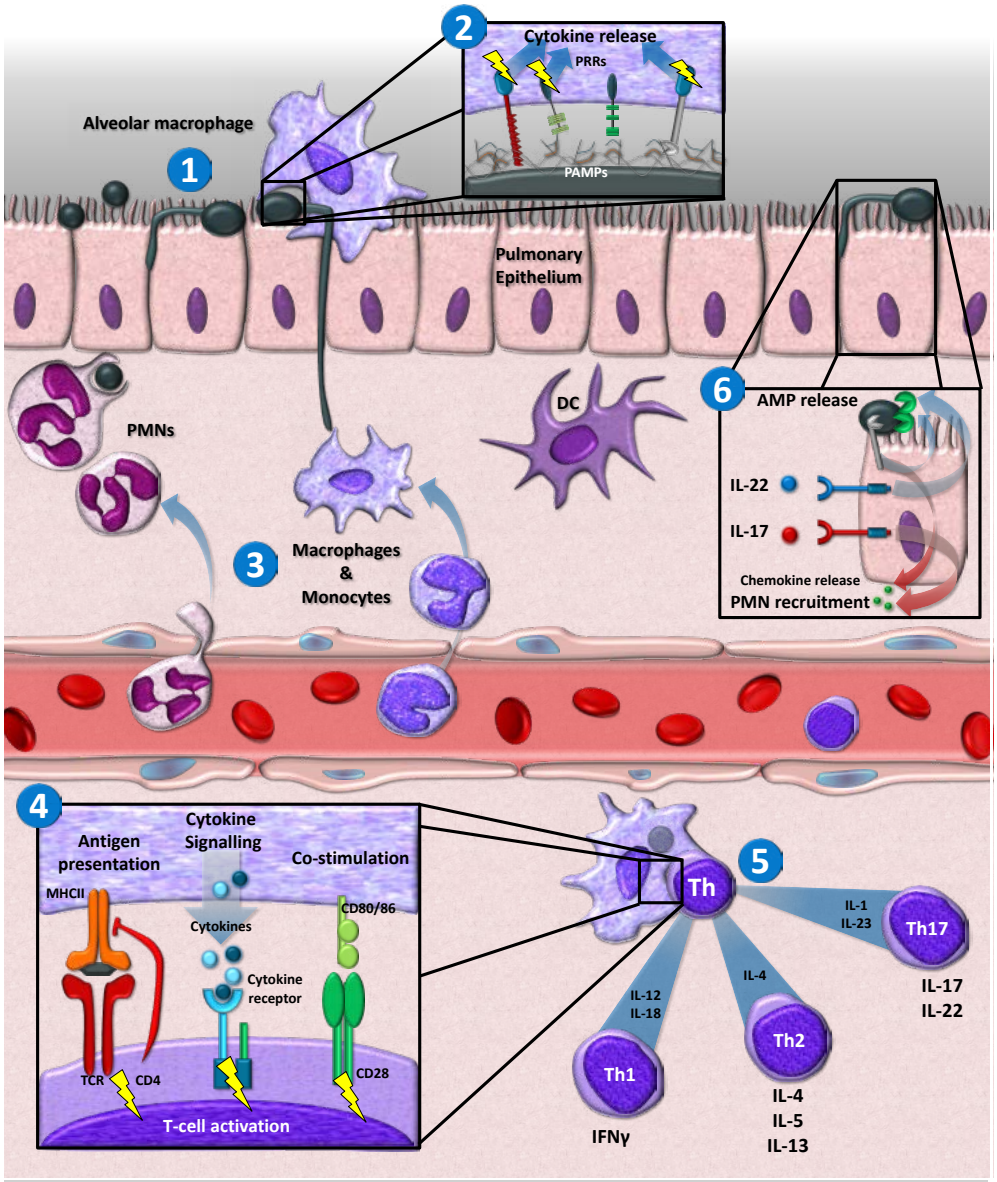


Figure 2| Schematic overview of the immune responses involved in the clearance *of A. fumigatus*
(1) The primary line of defence against *Aspergillus,* are the brush border pulmonary epithelium and alveolar macrophages that are capable of efficiently clearing inhaled conidia from the lungs. (2) When conidia are inefficiently cleared they can germinate into hyphae. These germinated conidia expose all kinds of pathogen associated molecular patterns (PAMPs) which can activate specific pattern recognition receptors (PRRs) leading to an induction of a cytokine and chemokine response. (3) Cytokines and chemokine released by the alveolar macrophages and pulmonary epithelium will induce recruitment and activation from polymorphonuclear cells (PMNs) or neutrophils and monocytes/macrophages. (4) After having recognized and phagocytosed fungal particles macrophages but also dendritic cells (DCs) can activate T-helper cells through antigen presentation, cytokine signalling and costimulatory molecules. (5) Depending on the cytokine profile different T-helper subtypes can be induced. (6) In particular the cytokines IL-17 and IL-22 released by Th17 cells can amplify the antifungal defence by the pulmonary epithelium by inducing the release of chemokines and antimicrobial peptides respectively.

Outline of the thesis

The previous paragraphs illustrate the disease spectrum of *A. fumigatus*, and demonstrate the importance of the immune response in preventing aspergillosis. However, many aspects of the host response and in particular the human host response remains unknown. The aim of this thesis is to study the pattern recognition and cytokine signalling pathways that orchestrate the human host response to *A. fumigatus* and to provide insights that may contribute to the prevention of *Aspergillus* infections and development of novel therapies.

The first section of the thesis **(chapter 2-6)**, entitled "*Induction of immune responses against Aspergillus fumigatus by pattern recognition receptors",* addresses the recognition mechanisms that play a role in various aspects of the host defence against *A. fumigatus*.

A relatively large number of studies have been conducted to elucidate which PRRs on the host immune cells play a role in the induction of the antifungal host defence against *A. fumigatus*. **Chapter 2** provides an overview of the literature on the PRRs that play a role in the recognition of *A. fumigatus*, and investigated how these receptors activate various antifungal host defence mechanisms to combat invasive *Aspergillus* infection.

The initiation of T-cell-mediated immune responses is highly dependent on the processing and presentation of antigens in MHC complexes. Autophagy is a mechanism that is involved in the recycling of cell organelles during cellular stress. However, autophagy is also involved in the host defence against various pathogens by facilitating antigen presentation [47]. During phagocytosis, a non-canonical autophagy pathway can be activated in which LC3 proteins are recruited to the phagosome (LC3-associated phagocytosis) which induces phagosome maturation and killing of phagocytosed pathogens [48]. The aim of **chapter 3** is to assess how this non-canonical autophagy pathway is induced by *Aspergillus fumigatus*. Numerous studies have demonstrated an increased immunogenicity of *Aspergillus* upon conidial swelling, through the increased exposure of β-glucan [49]. In this study we assessed the role of β-glucan exposure and its recognition by the dectin-1 and spleen tyrosine kinase (Syk) signalling pathway in LC3 recruitment to phagosomes containing *Aspergillus*. Moreover, we investigated whether corticosteroids, one of the immunosuppressive therapeutics that is highly associated with an increased susceptibility to aspergillosis [14,15,19], are able to interfere with induction of LC3-associated phagocytosis.

The PRRs that recognize *A. fumigatus* and orchestrate the induction of the immune response, will eventually lead to T-cell activation and induction of the adaptive immune responses. The cytokines IL-17, IL-22 and IFNγ, that have been primarily linked to Th17 and Th1 T-cell subsets, are known to play important roles in the host defence during invasive aspergillosis in mice. In **chapter 4** we studied these responses in human PBMCs. More specifically, we investigated which human lymphocyte subsets are involved in the production of these cytokines in response to *A. fumigatus,* and which PRRs and innate cytokines regulate the induction of these cytokines.

Besides Th1 and Th17 responses, T-cells can also become polarised towards the Th2 subset. The responses induced by this subset stand at the base of fungal sensitization and contribute to the

pathogenesis of ABPA, in individuals that mount a severe allergic Th2 responses, such as asthma and CF patients. To gain insight in the allergic response to *Aspergillus,* we investigated in **chapter 5** how *A. fumigatus* induces Th2 cytokine responses. We explore which pattern recognition pathways induce a Th2 bias, and investigated the ratio between protective Th1 responses and detrimental Th2 responses in patients with ABPA. Furthermore, we investigate how the Th2 response can be modulated by several immunomodulatory drugs that target the IL-1, tumour necrosis factor (TNF)α and IFNγ axis.

The NOD-like receptors are PRRs localised within the cytoplasm of innate immune cells. Thus far, they have been studied in the recognition of peptidoglycan moieties from the bacterial cell wall and are associated with Crohn's disease. However, there is increasing evidence that the NOD2 receptor is involved in recognition of *Aspergillus*. **Chapter 6** investigates how deficiency in the NOD receptors influences the host response against *A. fumigatus*, and investigates the role of both NOD1 and NOD2 in a mouse model for invasive aspergillosis in an immunosuppressed background.

The second section of this thesis **(chapter 7-12)**, entitled "*Cytokines involved in orchestrating the antifungal immune response"*, focuses on cytokines and modulation of cytokines that are involved in the antifungal immune response against *A. fumigatus*.

The IL-1 family cytokines are highly associated with host defence against bacterial and fungal pathogens by their capacity to induce neutrophil recruitment, regulate the induction of T-cell proliferation and skewing of the T-cell responses towards a Th17 response. A review of the current literature on the role of the IL-1 family cytokine response and in particular the cytokine IL-1β, in the host defence against *A. fumigatus* is presented in **chapter 7**.

Recently, the *Aspergillus* cell wall polysaccharide galactosaminogalactan has gained interest because of its immunomodulatory effects and its capacity to render mice susceptible to invasive aspergillosis [50]. **Chapter 8** describes the study that investigated how galactosaminogalactan modulates the host response against *Aspergillus.* Due to the previous observation that galactosaminogalactan inhibited neutrophil recruitment to the lungs of infected mice [50], we specifically investigated whether and how it modulates the IL-1 axis.

Common genetic variations in cytokine genes can affect their expression or function, thereby influencing the capacity to mount an effective host response. In **chapter 9** we investigated how polymorphisms within the genes encoding for IL-1β and IL-1Ra affected *Aspergillus*-induced cytokine responses. Furthermore, in a cohort of solid organ transplant patients we investigate whether polymorphisms in *IL1B, IL1RN* and the *DEFB1* gene, encoding for the antimicrobial peptide β-defensin-1, affect the susceptibility to invasive fungal infections.

For some of the members of the IL-1 cytokines the role in the immune system is still largely unknown. In **chapter 10** we introduce the IL-36 family, which is a subclass of the IL-1 family. Although these cytokines belong to the intensively studied IL-1 family, their role was unknown until recently. In this chapter, we give an overview on the knowledge about the biology of these cytokines and their role in a wide spectrum of diseases. The IL-36 cytokines have been found to

play an important role in recruitment of neutrophils to the lungs [51], therefore they gained our interest and we hypothesized that they could potentially play an important role in the host defence against *Aspergillus*. Therefore, we investigated in **chapter 11** whether IL-36 cytokines are induced by *Aspergillus*. Since the IL-36R is highly expressed on naive T-cells [52], we also assessed the role of IL-36 in *Aspergillus*-induced T-cell responses, and in particular the Th17 response which is highly associated with neutrophil recruitment.

Mortality due to fungal infections and especially aspergillosis is still very high, despite the introduction of azoles and echinocandins as novel therapeutic agents [53]. It is believed that an important reason for this is the immunosuppression often encountered in the patients, and it has been suggested that adjunctive immunotherapy can provide a necessary complementary step for the improvement of the outcome in these patients [54]. **chapter 12** describes a case series of patients with invasive fungal infection in whom we systematically addressed the immunological effects of IFNγ immunotherapy.

The findigs presented in this thesis that can potentially impact development of novel immunotherapy and genetic screening for susceptibility against aspergillosis are discussed in the general discussion in **chapter 13.**

A summary of the findings and conclusions of this thesis is presented in **chapter 14**.

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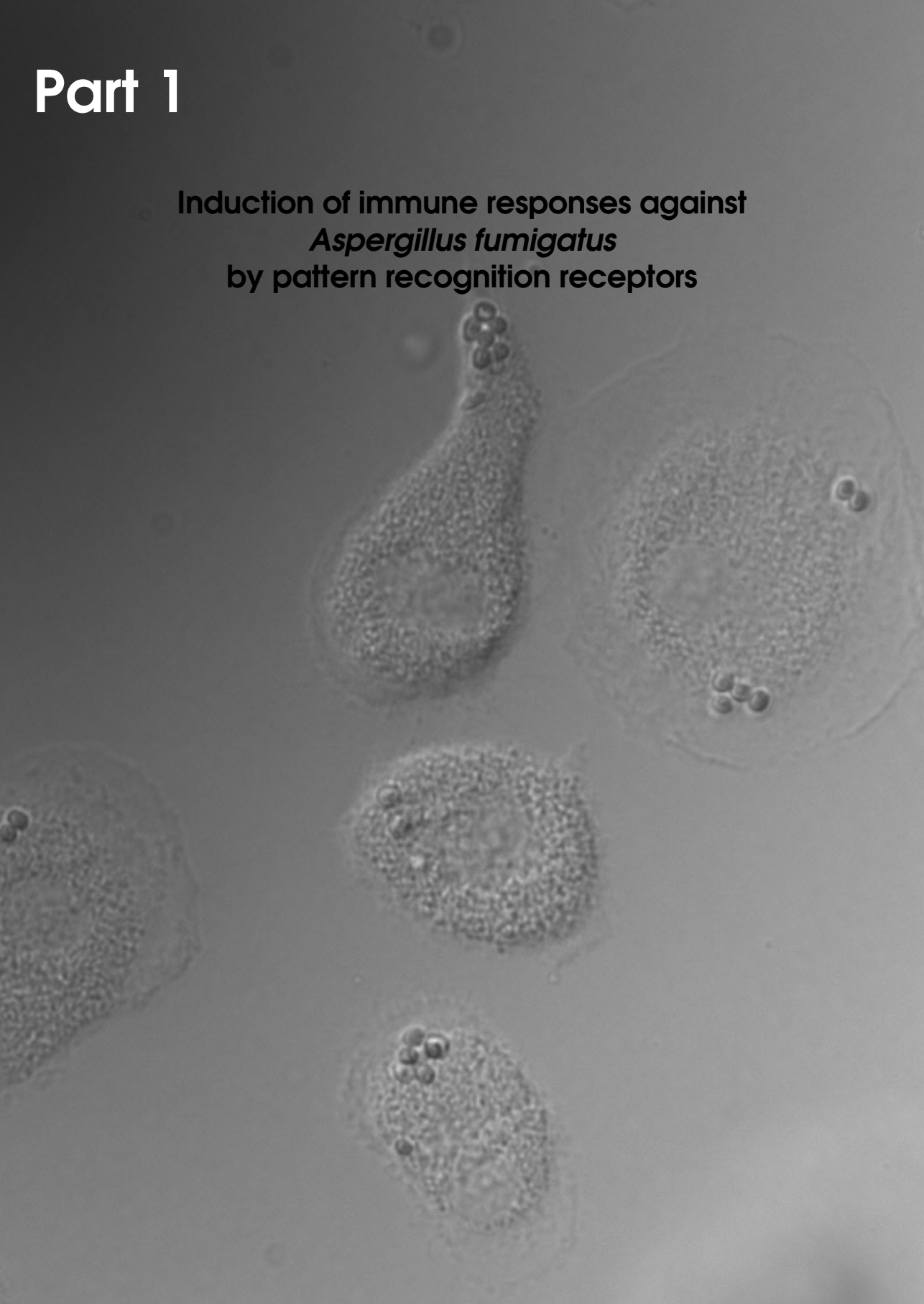
Part 1

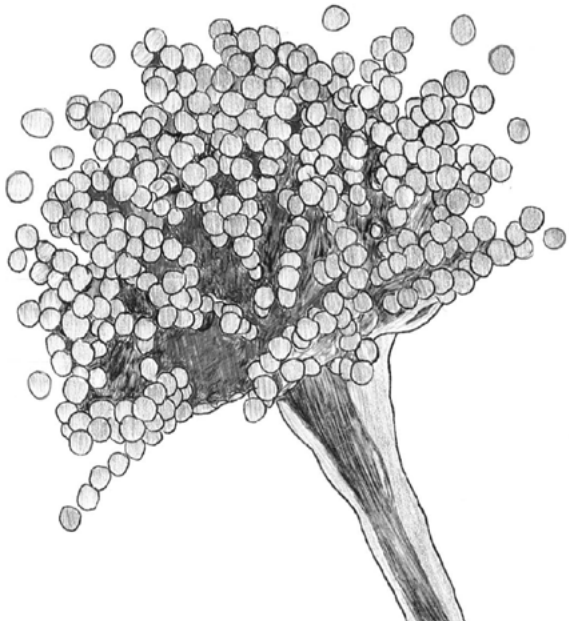
Induction of immune responses against *Aspergillus fumigatus* by pattern recognition receptors



Chapter 2

Pattern recognition receptors and their role in invasive aspergillosis

Mark S. Gresnigt, Mihai G. Netea, Frank L. van de Veerdonk



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Abstract

Pattern recognition receptors (PRRs) are germline receptors that recognize conserved structures on microorganisms. Several PRRs have been identified in the recent years that are involved in the immune response against *Aspergillus fumigatus*. The role of PRRs in invasive pulmonary aspergillosis becomes especially apparent in the setting of an immunocompromised status of the host, because of the redundancy of many PRRs in the host defense against *A. fumigatus*. Studies that investigated the PRRs and their effector pathways in invasive aspergillosis have led to a better understanding of the pathogenesis of invasive aspergillosis in immunocompromised patients. This knowledge may pave the way for novel diagnostic and immunomodulatory treatment strategies that are needed to overcome the high mortality associated with invasive *A. fumigatus* infection in immunocompromised patients.

Introduction

Aspergillus fumigatus can cause invasive pulmonary aspergillosis (IPA) in immunocompromised patients. The management of invasive aspergillosis still presents a major challenge, and knowledge of the host defence against *A. fumigatus* is essential to develop new treatment strategies. Here we review the literature on pattern recognition receptors (PRRs) that recognize *A. fumigatus* and focus on their role in the host defence against IPA.

Pattern recognition of *A. fumigatus*

The first step in the innate host defence against *A. fumigatus* is the recognition of fungal cell wall components by PRRs present on and in the cells of the innate immune system. Three of the four major families of PRRs have been implicated in recognition of *Aspergillus spp*: the Toll-like receptors (TLRs), the C-type lectin receptors (CLRs), and the nucleotide oligomerization domain (NOD)-like receptors (NLRs). RIG-I helicases are not currently known to be involved in *A. fumigatus* recognition (Figure 1).

Toll-like Receptors

Both TLR2 and TLR4 signalling are associated with NFκB translocation and production of pro-inflammatory cytokines in response to *A. fumigatus in vitro* [1,2]. Initial studies have shown that peritoneal macrophages deficient in TLR2 and MyD88 stimulated with *A. fumigatus* produce significantly lower tumour necrosis factor(TNF)α levels, whereas TLR4 deficient peritoneal macrophages produce normal levels of TNFα when stimulated with the fungus [3]. Blocking TLR4, but not TLR2, resulted in decreased *A. fumigatus*-induced TNFα production by human adherent monocytes [4]. Furthermore, conidia and hyphae of *A. fumigatus* stimulate cytokine production in macrophages through TLR2, whereas only conidia are able to stimulate macrophages by TLR4 [5]. These *in vitro* studies are the first reports that have demonstrated a role for TLRs in the recognition of *A. fumigatus*. Most of these studies controlled for the absence of bacterial LPS.

In a murine model of IPA, TLR2 and TLR4 deficiency are both correlated with decreased cytokine levels, and TLR2$^{-/-}$, TLR4$^{-/-}$ or MyD88$^{-/-}$ mice have a higher fungal burden than WT mice [6,7]. TLR2 and TLR4 are important for the recruitment of PMNs to the lung during IPA, and the optimal killing of *A. fumigatus in vivo* requires both TLR2 and TLR4 [7]. TLR2 can form heterodimers with TLR1 or TLR6, and a recent study has reported that TLR1$^{-/-}$ and TLR6$^{-/-}$ mice have increased fungal burdens during *A. fumigatus* infection. In addition, it has been shown that TLR1$^{-/-}$ and TLR6$^{-/-}$ macrophages produce less pro-inflammatory cytokines compared to wild type mice [8].

The intracellular TLRs, TLR3 and TLR9, have also been reported to play a role in anti-*Aspergillus* host defence. Epithelial cells can contribute to the protection against *A. fumigatus* in a TLR3 dependent pathway [9]. TLR3 signals through the adaptor molecule TRIF, and TRIF$^{-/-}$ mice are highly susceptible to IPA. Furthermore, *A. fumigatus* RNA is recognized by TLR3, and dendritic cells (DCs) can promote protective cytotoxic T cell responses through a TLR3-mediated pathway [9]. In line with these findings, TLR3$^{-/-}$ mice are highly susceptible to IPA [9]. TLR9 can recognize *A. fumigatus*

DNA [10], and TLR9 is actively being recruited to phagosomes that contain *A. fumigatus* conidia [11]. Interestingly, but still largely unexplained, is the finding that TLR9$^{-/-}$ mice are protected against lethal *A. fumigatus* infection [7].

A. fumigatus itself can influence the immune response by modulating TLR signalling. *A. fumigatus* conidia can decrease TLR2 expression on the surface of monocytes by inducing TLR2 internalization, while *A. fumigatus* hyphae can selectively downregulate the TLR4-mediated immune response [12]. Moreover, the *Aspergillus* cell wall components β-glucan and galactomannan can suppress TLR4-induced responses, while α-glucan inhibits TLR2- and TLR4-induced interleukin(IL)-6 production [13]. TLR5 is strongly upregulated by *A. fumigatus* conidia in human monocytes, and although there is no evidence for direct recognition of *A. fumigatus* by TLR5, activation of TLR5 results in decreased capacity of monocytes to inactivate viable *A. fumigatus* conidia [14]. This suggests that the increased expression of TLR5 induced by *A. fumigatus* on the surface of human monocytes can help survival of

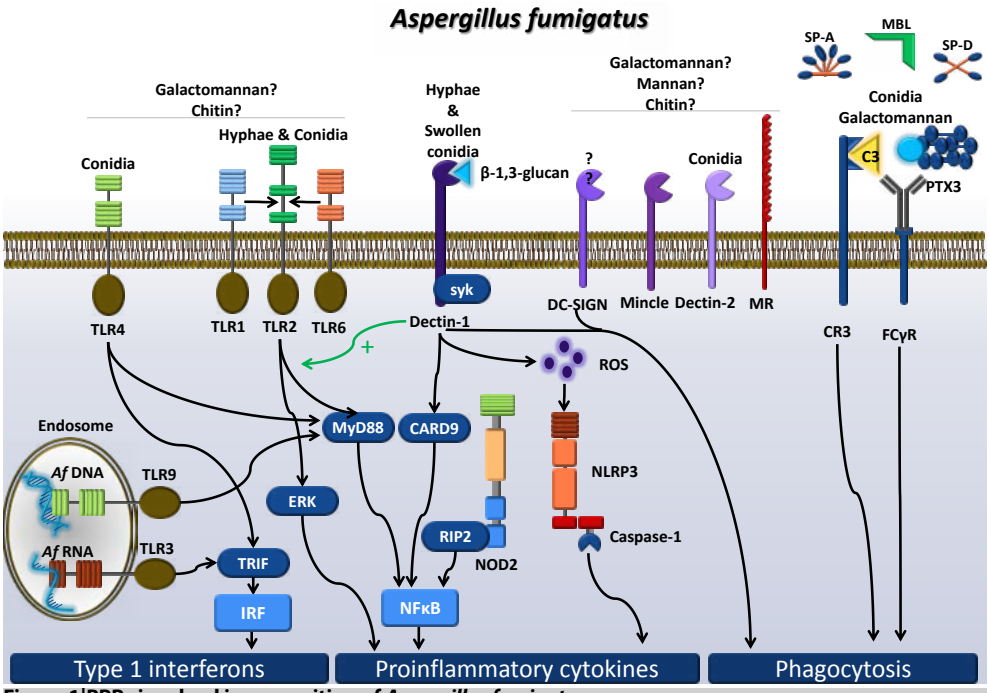

Figure 1|PRRs involved in recognition of *Aspergillus fumigatus*
Schematic overview of the pattern recognition receptors (PRRs) and the downstream signalling pathways involved in the recognition of *A. fumigatus*. Pathogen associated molecular patterns (PAMPs) of *Aspergillus* lead to various host responses such as cytokine production and phagocytosis through the activation of PRRs. TLR (Toll-like receptor), Syk (spleen tyrosine kinase), DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin), MR (Mannose Receptor), CR3 (complement receptor 3), C3 (complement factor C3), SP-A/D (Surfactant Protein-A/D), PTX-3 (pentraxin-3), FCγR (FC gamma receptor), *Af* (*Aspergillus fumigatus*), TRIF (TIR-domain-containing adapter-inducing interferon-β), IRF (Interferon regulating factor), ERK (extracellular-signal-regulated kinase), MYD88 (Myeloid differentiation primary response gene 88), CARD9 (Caspase recruitment domain-containing protein 9), RIP2 (receptor interacting protein 2), NFκB (nuclear factor kappa-light-chain-enhancer of activated B-cells), NOD2 (Nucleotide-binding oligomerization domain-containing protein 2), NLRP3 (NACHT, LRR and PYD domains-containing protein 3), ROS (reactive oxygen species).

the fungus when ligands are present that activate TLR5. However, all TLR5 ligands known to date are of bacterial origin, and TLR5 is most likely influencing host defence against *Aspergillus* in the context of co-colonization or co-infection with bacteria.

In line with the *in vitro* and *in vivo* data demonstrating an important role for TLRs in anti-*Aspergillus* host responses, several studies have reported polymorphisms in TLRs that are associated with invasive aspergillosis in patients. A single nucleotide polymorphisms (SNP) in TLR4 that influences TLR4 function is associated with invasive aspergillosis in patients that received heamatological stem cell transplantation (HSCT) [15,16]. Although, functional studies have demonstrated involvement of TLR2 in the recognition of *A. fumigatus,* to date no polymorphisms in TLR2 have been associated with increased susceptibility to *A. fumigatus* infection. However, SNPs in TLR1 and TLR6, co-receptors that form heterodimers with TLR2, are associated with the development of invasive aspergillosis [17]. A TLR3 SNP has been reported to be associated with increased risk of IPA in a cohort of patients with HSCT [9].

C-type lectin receptors

The extracellular portion of CLRs consists of several carbohydrate recognition domains, which enable binding to sugar moieties present on microorganisms. Dectin-1 recognizes β-1,3-glucans and is involved in the recognition of *A. fumigatus* [18]. Dectin-1-binding to germ tubes augments TLR2-mediated production of pro-inflammatory cytokines [19]. Alveolar macrophages induce strong inflammatory responses to swollen and germinating conidia, which correlates with the increased surface expression of β-glucan during germination [20]. The importance of dectin-1 for anti-*Aspergillus* host defence has been demonstrated by the fact that dectin-1 deficient mice are more susceptible to IPA [18,21]. *A. fumigatus* induces the cytokines IL-17 and IL-22, important for neutrophil recruitment and the production of defensins by epithelial cells respectively, in a dectin-1 dependent way [22-24]. It must, however, be noted that the current literature describes both protective and detrimental effects of IL-17 in the host, depending on the host conditions in which the *Aspergillus* infection occurs [21,24,25]. Human airway epithelium and DCs also express dectin-1, which enables them to recognize *A. fumigatus* [26]. Human monocytes with the dectin-1 Tyr238X polymorphism that results in an early stop codon produce less pro-inflammatory cytokines in response to *A. fumigatus* stimulation. Importantly, this polymorphism correlates with an increased susceptibility to develop IPA in patients receiving HSCT [26,27]. Recently, a study demonstrated increased susceptibility to invasive aspergillosis in haematological patients that carried SNPs in dectin-1 intronic regions [28].

Dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) is a CLR that is expressed on DCs and macrophages, and has been shown to be involved in binding and internalization of *A. fumigatus* [29]. Co-stimulation of neutrophils with *Aspergillus*-stimulated DCs results in the upregulation of co-stimulatory molecules on DCs, a process that is dependent on DC-SIGN [30]. Notably, SNPs in intronic regions or in the 3'-untranslated region(UTR) of the DC-SIGN gene have been associated with increased susceptibility to invasive aspergillosis [28]. Another CLR, the mannose receptor (MR) has been suggested to be involved in the induction of pro-inflammatory cytokine

production induced by *A. fumigatus* conidia that are deficient in melanin [31]. Dectin-2 mediates the release of cysteinyl leukotrienes from murine bone marrow derived DCs that are stimulated with extracts from *A. fumigatus*, suggesting that dectin-2 recognizes *A. fumigatus* [32]. However, the function and the importance of the MR and dectin-2, as well as the role of other CLRs in anti-*Aspergillus* host defence such as mincle or macrophage galactose lectin (MGL), remains to be determined.

NOD-like receptors

In addition to the TLRs and CLRs, NOD-like receptors (NLRs) that are localised intracellular and recognize intracellular PAMPs have been reported to be involved in the recognition of *Aspergillus*. NLRs are generally subdivided in two categories: firstly, NOD1 and NOD2 are the main intracellular receptors for peptidoglycans, whereas the function of the members of the NLRP subfamily is mainly in the formation of the inflammasome. NOD2 is upregulated after exposure to *A. fumigatus*, and recently it has been reported that NOD2 recognizes *A. fumigatus,* which subsequently results in the activation of NFκB [33]. However, individuals completely deficient in NOD2 and suffering from Crohn's disease [34] do not show an increased susceptibility to invasive aspergillosis.

Nlrp3 is an NLR that has been extensively studied over recent years. Nlrp3 is involved in the activation of the inflammasome, which is a protein complex that can activate caspase-1. Caspase-1 is an enzyme that can cleave the inactive protein pro-IL-1β into the active cytokine IL-1β. *A. fumigatus* hyphae, but not conidia, can activate the NLRP3 inflammasome through the induction of reactive oxygen species (ROS) and potassium efflux [35]. This in turn results in the release of active IL-1β. This process is spleen tyrosine kinase(Syk)-dependent, which is an adaptor molecule downstream of dectin-1, suggesting that dectin-1 is involved in the induction of ROS, the activation of the NLRP3 inflammasome, and release of IL-1β induced by *A. fumigatus*.

Soluble pattern recognition receptors

Collectins are soluble C-type lectins that bind to glycoconjugated structures. Surfactant proteins A (SP-A) and D (SP-D) are collectins that are produced by pneumocytes and can bind to *A. fumigatus* conidia. The survival of immunosuppressed mice infected with *A. fumigatus* is significantly increased when mice are treated with SP-D [36]. In line with this, SP-D$^{-/-}$ mice die earlier then WT mice, and treatment of SP-D$^{-/-}$ mice with SP-D decreases mortality from 50% to 33% [37]. Interestingly, SP-A$^{-/-}$ mice are less susceptible to invasive aspergillosis and SP-A treatment increases mortality in SP-A$^{-/-}$ mice [37].

Mannose-binding lectin (MBL) can bind to *A. fumigatus,* but this does not necessarily result in enhanced killing of *A. fumigatus* [38]. Immunocompetent mice deficient in MBL are protected against invasive aspergillosis [39]. In sharp contrast, immunosuppressed mice with invasive aspergillosis that are treated with recombinant MBL have a clear survival benefit [38]. Thus, MBL could have different effects depending on the status of the immune system. In addition, low MBL concentrations in serum are associated with higher susceptibility to invasive aspergillosis [40].

Pentraxin 3 (PTX3) is a soluble pattern recognition receptor that can bind to *Aspergillus* conidia, but not to hyphae [41]. PTX3 deficient neutrophils display impaired phagocytosis of *Aspergillus* [41].

Dendritic cells (DCs) and alveolar macrophages can recognize *A. fumigatus* through a PTX3-dependent pathway and genetic deletion of PTX3 in mice results in impaired recognition of *A. fumigatus* and increased susceptibility to *A. fumigatus* infection [41]. The complement receptor 3 (CR3) and the FCγR are required for recognition of PTX3-opsonized *Aspergillus* [42]. Notably, NADPH-dependent ROS deficient mice [43], and corticosteroid immunosuppressed rats [44], which are both highly susceptible to *Aspergillus* infection, can be rescued by PTX3 treatment. Therefore, PTX3 treatment can prove to be an important immunotherapeutic strategy in invasive aspergillosis.

Pattern recognition receptors orchestrate the immune response

When *A. fumigatus* conidia are inhaled and reach the alveolus, PRRs present on alveolar macrophages and epithelial cells, and the soluble PRRs from the alveolar fluid will recognize the fungus and induce the activation of the immune response (Figure 2). Alveolar macrophages and epithelial cells will release cytokines and chemokines in response to *A. fumigatus* recruiting innate immune cells, a process dependent on TLR2, TLR4 and dectin-1 [1-7]. Both neutrophils and macrophages recognize and phagocytose *A. fumigatus* conidia, a process that can be enhanced by soluble PRRs such as PTX3 and possibly SP-D and MBL [36,38,42], and kill conidia by NADPH-dependent ROS production [45,46]. *A. fumigatus*-induced ROS production can also trigger the NLRP3 inflammasome through dectin-1/Syk signalling, resulting in the release of active IL-1β and recruitment of neutrophils [35]. Neutrophils will release neutrophil entrapment traps (NETs) that in turn might help to kill and inhibit growth of *A. fumigatus* hyphae that will be too large to be phagocytized [47,48]. Notably, restoration of NADPH oxidase activity by gene therapy in patients with chronic granulomatous disease provides protection against invasive aspergillosis [49,50]. The restoration of the NADPH oxidase activity restores NET formation and calprotein release, which is associated with more efficient killing of *Aspergillus ex vivo* [49,50]. Recently, plasmacytoid DCs have been reported to have the ability to kill hyphae extracellularly [51]. However, the PRRs involved in this process, and the PRRs that trigger the formation of NETs in response to *Aspergillus* have not yet been elucidated. If the innate immune system fails to control the infection, antigen-presenting cells such as dendritic cells will activate the adaptive immune response, a process where dectin-1 and TLR3 play an important role [9,52].

The interplay between other CLRs and TLR-MyD88 pathways are also important for the induction of the adaptive immune response. Especially dectin-1, which can regulate the balance between Th1 and Th17 responses [53]. Although TLR-MyD88 signalling is crucial for interferon(IFN)γ production in *Aspergillus*-specific CD4 T-cells, it is not required for the recruitment or proliferation of these cells in response to *A. fumigatus* [54]. The TLR-MyD88 pathway plays a less important role in the induction of Th2 responses by *A. fumigatus* [54], in contrast to the induction of Th2 cells by OVA, which is MyD88 dependent [55].

Concluding remarks

Although PRRs play an important role in the host defence against *A. fumigatus*, it has to be noted that invasive *A. fumigatus* infections only occurs in immunosuppressed patients. MyD88$^{-/-}$ mice only develop invasive aspergillosis when they are immunosuppressed, and non-immunosuppressed patients with MyD88 deficiency are not prone to develop invasive pulmonary aspergillosis [56,57]. Furthermore, humans that lack surface expression of dectin-1, which is involved in many

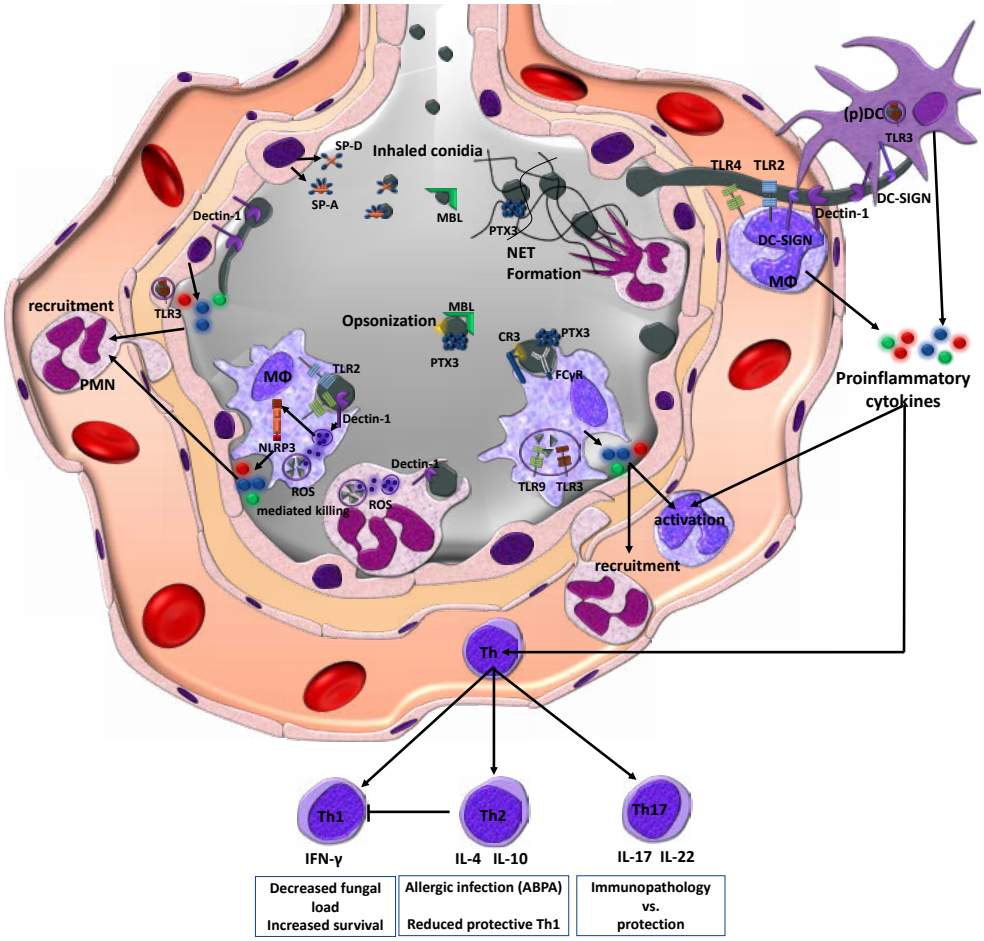

Figure 2|Effector functions of PRRs in anti-*Aspergillus* host defence
Overview of the PRR-mediated effector functions of innate immune cells that play a role in the pulmonary antifungal host defence against *A. fumigatus*. Inhaled conidia are recognized and opsonized by soluble PRRs like pentraxin 3 (PTX3), and mannose-binding lectin (MBL), and surfactant Protein-A/D (SP-A and SP-D). Epithelial cells and macrophages produce cytokines through Toll-like receptors (TLRs) that mediate recruitment of innate immune cells like neutrophils (PMNs) and dendritic cells (DCs). Following internalization by dectin-1 phagocytes induce reactive oxygen species (ROS) to mediate eradication of the internalized conidia. Neutrophils generate neutrophil extracellular traps (NETs) by releasing their intracellular contents and DNA. DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin), CR3 (complement receptor 3), FCγR (FC gamma receptor), NLRP3 (NACHT, LRR and PYD domains-containing protein 3).

fundamental anti-*Aspergillus* host responses, do not suffer from invasive aspergillosis when they are not immunosuppressed. These findings suggest that the TLR and dectin-1 pathways are redundant in anti-*Aspergillus* host defence. On the one hand, this suggests that only concomitant deficiencies in more recognition pathways at the same time will likely result in an increased susceptibility, and this hypothesis is supported by the increased susceptibility to infections in CARD9-deficient patients, who display defects in several CLR pathways, and possible also NOD2-dependent signalling [58]. On the other hand, invasive aspergillosis especially develops in patients that are immunosuppressed, and it is in this particular setting that polymorphisms and deficiencies in TLR-, dectin-1- and other PRR-signalling pathways become highly relevant for the antifungal host defence.

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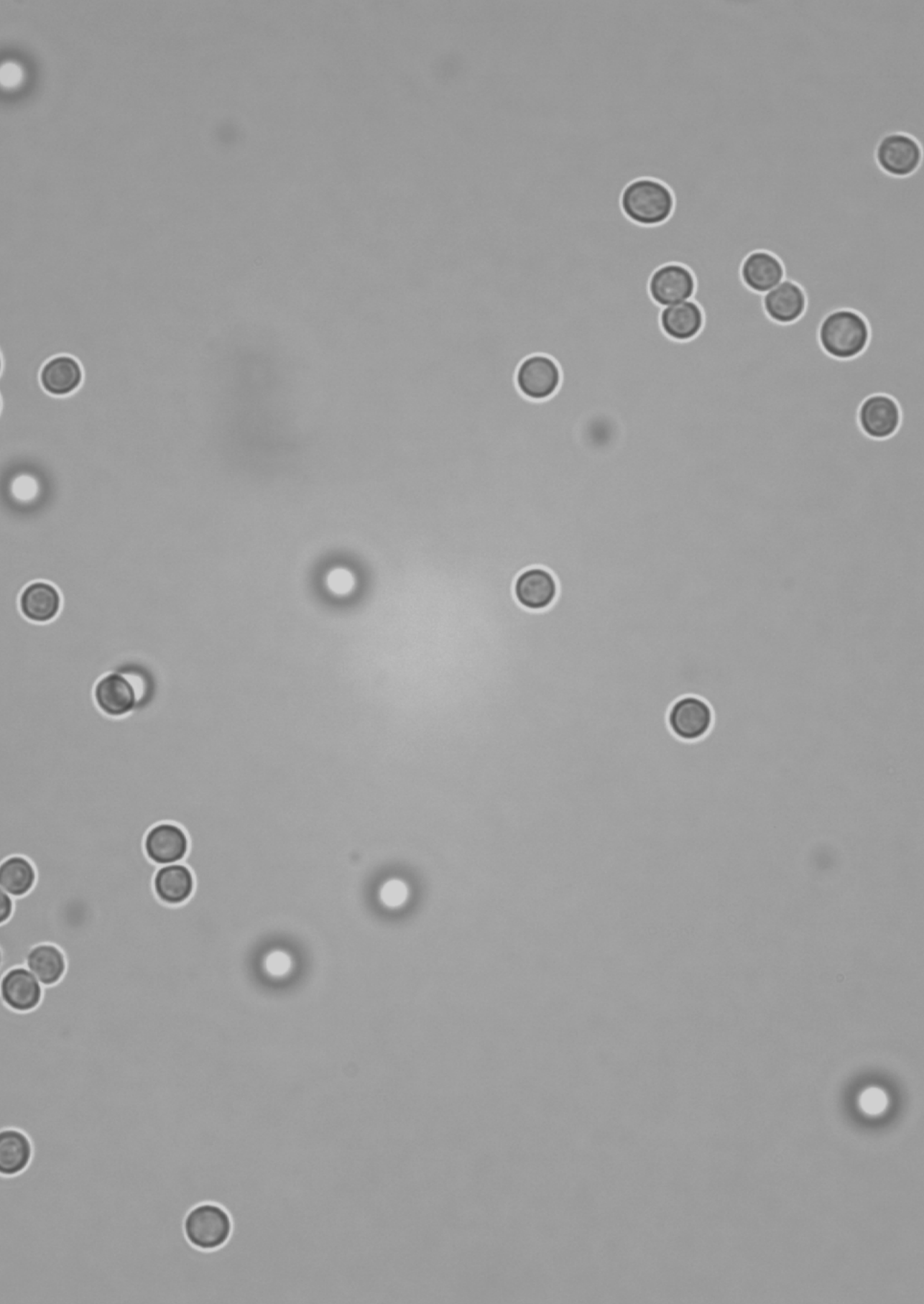

Chapter 3

Corticosteroids block autophagy protein recruitment in *Aspergillus fumigatus* phagosomes via targeting dectin-1/Syk kinase signalling

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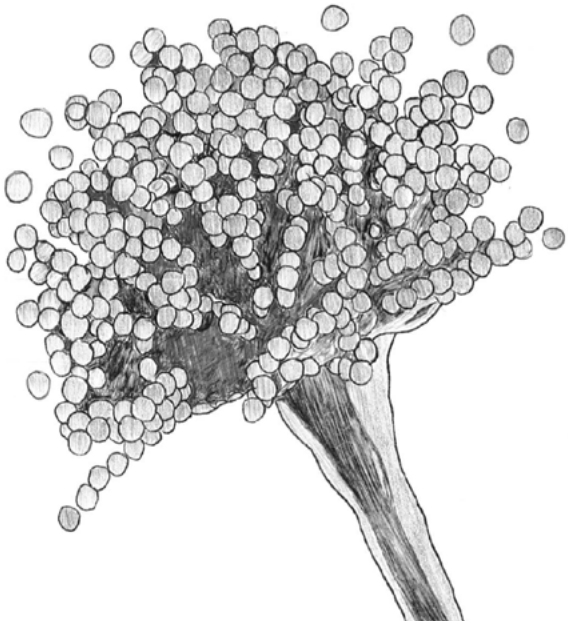

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Abstract

Aspergillus fumigatus is the predominant airborne fungal pathogen in immunocompromised patients. Genetic defects in NADPH oxidase, such as chronic granulomatous disease (CGD), and corticosteroid-induced immunosuppression lead to impaired killing of *A. fumigatus* and unique susceptibility to invasive aspergillosis (IA) via incompletely characterized mechanisms. Recent studies link Toll-like receptor activation with phagosome maturation via the engagement of autophagy proteins. In this study, we found that infection of human monocytes with *A. fumigatus* spores triggered selective recruitment of the autophagy protein LC3II in phagosomes upon fungal cell wall swelling. This response was induced by surface exposure of β-glucan and was mediated by activation of the dectin-1. LC3II recruitment in *A. fumigatus* phagosomes required spleen tyrosine kinase (Syk)–dependent production of reactive oxygen species and was nearly absent in monocytes of patients with CGD. This pathway was important for control of intracellular fungal growth, as silencing of ATG5 resulted in impaired phagosome maturation and killing of *A. fumigatus*. *In vivo* and *ex vivo* administration of corticosteroids blocked LC3II recruitment in *A. fumigatus* phagosomes via rapid inhibition of phosphorylation of Src and Syk kinases and downstream production of reactive oxygen species. Our studies link dectin-1/Syk signalling with autophagy-dependent maturation of *A. fumigatus* phagosomes and uncover a potential mechanism for development of IA in the setting of CGD and corticosteroid-induced immunosuppression.

Introduction

Aspergillus fumigatus, a ubiquitous saprophytic fungus, is a leading cause of morbidity and mortality in immunocompromised patients [1]. Acquired quantitative and qualitative innate immune defects, typically encountered in haematological malignancy patients with severe chemotherapy-induced neutropenia and recipients of transplants following treatment with high doses of corticosteroids, are major predisposing factors for development of invasive aspergillosis (IA)[1-3]. *A. fumigatus* is currently regarded as an emerging fungal pathogen in a broad range of non-neutropenic hosts who receive prolonged courses of corticosteroid therapy [4], including patients with autoimmune and inflammatory diseases, and prolonged stay in intensive care units [1,4-6]. Moreover, patients with chronic granulomatous disease (CGD), a rare primary immunodeficiency characterized by genetic defects in NADPH oxidase complex, are uniquely susceptible to development of IA [1,2].

Although risk factors for development of IA are well characterized, the immunopathogenesis of this frequently lethal opportunistic mycosis is incompletely understood. In immunocompetent individuals, professional phagocytes, including resident alveolar macrophages, circulating monocytes, and neutrophils, efficiently eliminate *A. fumigatus* spores, which are inhaled in a daily basis, to prevent germination of spores to hyphae and development of invasive fungal disease [1,2,7,8]. *A. fumigatus* spores are degraded within acidified lysosomal compartments of human phagocytes via the complex process of phagolysosomal fusion [9,10]. Genetic defects in NADPH oxidase-derived reactive oxygen species (ROS) generation and corticosteroid therapy are associated with impaired maturation of *A. fumigatus* phagosomes and attenuated fungal killing, via incompletely characterized mechanisms [11-13].

The past few years have witnessed major advances in understanding innate sensing of fungi. Initial studies demonstrated that *A. fumigatus* preferentially activates TLR2 and TLR4 and results in NFκB-mediated immune responses [14,15]. Recent evidence suggests an emerging role for dectin-1 and other C-type lectin receptors (CLRs) in antifungal immunity [16-21]. Dectin-1 recognizes β-glucan carbohydrates in the fungal cell walls and triggers intracellular signalling via a cytoplasmic ITAM-like motif via recruitment of spleen tyrosine kinase (Syk) and Raf-1 kinase [16,22].

In contrast to the well-characterized role of pattern recognition receptors (PRRs) in activating signalling pathways for induction of cytokine release, their contribution in phagosome maturation is less well defined. Recently, the recruitment of proteins of the autophagy machinery, including LC3II, ATG5, and ATG7, in phagosomes containing microbial ligands in response to TLR activation was found to be important for phagolysosomal fusion and pathogen elimination by murine macrophages [23]. Although the signalling regulating autophagy protein recruitment in TLR-containing phagosomes has not been characterized, this response was shown to be dependent on NADPH-derived ROS production [24]. At present, there is no clear evidence on whether and how innate sensing of *A. fumigatus* is linked to phagosome maturation and killing by professional phagocytes.

In this study, we found that *A. fumigatus* infection of human monocytes triggered a selective recruitment of LC3II autophagy protein in phagosomes upon fungal cell wall swelling. This response was induced by surface exposure of β-glucan, required activation of dectin-1/Syk/ROS signalling,

and it was nearly absent in monocytes of patients with CGD. This pathway was important for fungal clearance because conditional inactivation of ATG5 resulted in attenuated phagolysosomal fusion and killing of *A. fumigatus* spores. Importantly, *in vivo* or *ex vivo* treatment of human monocytes with hydrocortisone blocked LC3II recruitment in *Aspergillus*-containing phagosomes via rapid inhibition of phosphorylation of Src and Syk kinases and subsequent blockade in ROS production. Overall, our studies link dectin-1/Syk signalling with autophagy-dependent maturation of fungal phagosomes and uncover a potential target for development of novel immunotherapies against invasive *Aspergillus* infections.

Experimental procedures

Reagents | Highly purified *Escherichia coli* LPS (catalog number 437627) was purchased from Calbiochem; Laminarin from *Laminaria digitata* (catalog number L9634), β-1,3-D-glucanase from *Aspergillus niger* (catalog number 49101), and 2′,7′-dichlorofluorescin diacetate (DCFH-DA; catalog number D6883) were all obtained from Sigma Aldrich. Purified particulate β-glucan (curdlan) was from Waiko (Tokyo, Japan). Yeast whole glucan particles (WGP) were from Biothera. For immunofluorescence imaging studies, WGP was labelled with fluorescein dichlorotriazine (Molecular Probes-Invitrogen). Anti-β-(1,3)-Glucan mAb (catalogue number 400-2) was from Biosupplies (Parkville, Australia). Blocking mAb for dectin-1 (GE2) (catalogue number ab82888; 10 mg/mL) was from Abcam. TLR2 (10 mg/mL), TLR4 (10 mg/mL), and appropriate isotype control Abs were from eBioscience. In some experiments, highly purified *Bartonella quintana* LPS was used as a potent TLR4 inhibitor. A Syk inhibitor (1 mM; catalogue number 574711), piceatannol (40 mM; catalog number 527948), and raf-1 inhibitor (40 mM; catalog number 553008, GW5074) were from Calbiochem. Hydrocortisone (Lyocortin) was from Vianex. Anti-LC3 Ab used for immunofluorescence was from Nanotools (0231-100/LC3-5F10). FITC-conjugated dectin-1 Ab (MCAA4661FT) was from AbD Serotec. Latex beads of 3 μm diameter were purchased from Sigma Aldrich. Coating of latex beads with IgG or BSA was performed by overnight rotating incubation at 4°C with human IgG (1 mg/mL) or BSA (1 mg/mL) followed by three washes with PBS.

Isolation and stimulation of human primary cells from patients and controls | Healthy volunteers without any known infectious or inflammatory disorders donated blood as a control group for the assessment of LC3II recruitment in fungal phagosomes. In addition, PBMCs were isolated from three patients with CGD harbouring homozygous mutations in the NCF1 gene ($p47^{phox}$) in which the complete absence of ROS production has been demonstrated, and three homozygous patients with the early stop codon mutation Tyr238X in dectin-1 (dectin-$1^{-/-}$). After informed consent, blood was collected by venipuncture from these patients and volunteers into 10 mL EDTA tubes. Six consecutive patients with various rheumatologic diseases receiving treatment with a standard dose of corticosteroids (Table I) were recruited from the Rheumatology Department, University Hospital of Heraklion. Monocytes from healthy controls and patients were isolated from PBMCs

using magnetic bead separation with anti-CD14-coated beads (MACS; Miltenyi Biotec) according to the protocol supplemented by the manufacturer. The monocytes were resuspended in RPMI 1640 culture medium supplemented with 1% gentamicin, 1% L-glutamine, and 1% pyruvate. The cells were counted in a Bürker counting chamber, and their number was adjusted to $1x10^6$/mL. A total of 23105 monocytes per condition in a final volume of 200 mL were allowed to adhere to glass coverslips (Ø12 mm) for 1 hour, after which they were exposed to *A. fumigatus* spores at a multiplicity of infection (MOI) of 3:1 at 37°C for 1 hour. After stimulation, the coverslips were washed twice with PBS to remove medium, and nonphagocytosed spores and cells were fixed on the coverslips for 15 minutes in 4% paraformaldehyde (PFA). Subsequently, the coverslips were washed with PBS followed by a fixation in ice-cold methanol for 10 minutes in -20°C, after which coverslips were stored in PBS at 4°C until immunofluorescence staining was performed.

Microorganisms and culture conditions | The *A. fumigatus* strains Af293 (ATCC 46645) and the GFP-*A. fumigatus* strain (kind gift of K. A. Marr) were used in this study. All isolates were grown on YAG agar plates for 3 days at 37°C. Fungal spores in the presence of sterile 0.1% Tween-20 in PBS were harvested by gentle shaking, washed twice with PBS, filtered through a 40 µm pore size cell strainer (Falcon) to separate conidia from contaminating mycelium, counted by a haematocytometer, and suspended at a concentration of 10^8 spores/mL. Swollen *A. fumigatus* spores were obtained following growth in liquid RPMI 1640 media for 4-6 hours at 37°C. Typically, 90% of fungal spores were visibly swollen. The conidia were labelled with FITC as previously described [9]. Briefly, freshly harvested conidia $5x10^7$/mL 50 mM Na carbonate buffer (pH 10.2) were incubated with FITC at a final concentration of 0,1 mg/mL at 37°C for 1 hour and washed by centrifugation three times in PBS- 0,1% Tween 20.

Enzymatic digestion of β-glucan in swollen *A. fumigatus* spores was performed by using β-1,3-D-glucanase (Sigma Aldrich). *A. fumigatus* spores were incubated overnight in a water bath with 100 U/mL β-glucanase at a temperature of 55°C and pH 5. Inactivation of enzyme was achieved by 10 minutes incubation at 100°C followed by three washes in PBS. Verification of β-glucan digestion was performed by immunostaining with an anti-β-glucan mAb. Inactivation of fungi was done by heat exposure (30 minutes, 65°C) or exposure to 1% PFA (4°C, overnight) following by treatment with glycine (100 mM/PBS) and three washes in PBS. PFA inactivation of *A. fumigatus* spores had no effect on β-glucan surface exposure as evidenced by immunostaining.

Immunofluorescence staining | For immunofluorescence imaging, cells were seeded in coverslips pretreated with polylysine, fixed with 4% PFA for 15 minutes in room temperature following by 10 minutes of fixation with ice-cold methanol at 220°C, washed twice with PBS, permeabilized by using 0,1% saponin (Sigma Aldrich), blocked for 30 minutes in PBS plus 2% BSA, incubated for 1 hour with a mouse mAb to LC3 (1:50; Nanotools), washed twice in PBS plus 2% BSA, and stained by a secondary Alexa^{555} goat anti-mouse Ab (1:500; Molecular Probes), followed by DNA staining with 10 mM TO-PRO-3 iodide (642/661; Invitrogen). After the washing steps, slides were mounted in Prolong Gold

antifade media (Molecular Probes). Images were acquired using a laser-scanning spectral confocal microscope (TCS SP2; Leica Microsystems), LCS Lite software (Leica Microsystems), and a 403 Apochromat 1.25 NA oil objective using identical gain settings. A low fluorescence immersion oil (11513859; Leica Microsystems) was used, and imaging was performed at room temperature. Unless otherwise stated, mean projections of image stacks were obtained using the LCS Lite software and processed with Adobe Photoshop CS2 (Adobe Systems).

Phagosome acidification was assessed by use of the acidotropic dye LysoTracker Red DND-99 according to the manufacturer's instructions (Invitrogen) and immunostaining with a mouse mAb to CD63 (catalogue number 556019; BD Pharmingen) in primary human monocytes and THP- 1 cells. THP-1 cells (American Type Culture Collection) were maintained in complete medium containing RPMI 1640 supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 100 U/mL penicillin, 100 mg/mL streptomycin, 0.05 mM 2-ME, 4.5 g/L glucose, and 10% FCS v/v) at 37°C (5% CO_2), with passage every 3 days. Briefly, for LysoTracker staining, THP-1 cells were seeded on coverslips in 24-well flat-bottom plates and differentiated to macrophages following 48-h exposure to PMA (100 mg/mL; Sigma Aldrich) in RPMI 1640-10% FCS media. Cells were preloaded with LysoTracker, diluted 1:20,000 (v/v) in RPMI 1640 complete medium, for 2 hours and were subsequently infected at 4°C with FITC-labelled *A. fumigatus* conidia (MOI 5:1) in fresh medium without LysoTracker. After removal of nonphagocytosed conidia by washing with warm RPMI 1640 media, medium with LysoTracker was added to each well, and conidia internalization was initiated at 37°C. Infection was stopped after 2 hours, and the cells were washed with PBS, mounted on microscope slides, and examined immediately under the confocal microscope.

For β-glucan immunostaining of *A. fumigatus*, live or PFA-inactivated spores ($2x10^7$/ condition) were pelleted in propylene tubes, washed twice with PBS, blocked for 30 minutes in PBS plus 2% BSA, incubated for 1 hour with a mouse mAb to linear)-β-1,3-glucan (Biosupplies; 1 mg/mL) at room temperature, washed twice in PBS plus 2% BSA, stained by a secondary Alexa Fluor^{555} goat anti-mouse Ab (Molecular Probes), and images were acquired by confocal microscopy.

Immunoelectron LC3 microscopy in monocytes | Immunoelectron microscopy was performed using mouse monoclonal LC3 Ab (Nanotools), applying the pre-embedding gold enhancement method as described previously [25]. Primary human monocytes cultured on polylysine pretreated coverslips were fixed with 4% PFA (Nacalai Tesque) for 15 minutes at room temperature. After washing with the same buffer three times for 5 minutes, the fixed cells were permeabilized using 0,25% saponin in PBS. The cells were washed with PBS, blocked by incubating for 30 minutes in PBS containing 0,1% saponin, 10% BSA, 10% normal goat serum, and then exposed overnight to 0,01 mg/mL anti-LC3 mouse mAb or 0,01 mg/mL rat serum in the blocking solution. After washing with PBS containing 0.005% saponin, the cells were incubated with colloidal gold (1,4 nm diameter; Nanoprobes)-conjugated goat anti-mouse IgG in the blocking solution for 2 hour. The cells were then washed with PBS and fixed with 1% glutaraldehyde in PBS for 10 minutes. After washing with 50 mM glycine in PBS, 1% BSA in PBS, and finally with milliQ water (Millipore), gold labelling was intensified

with a gold enhancement kit (GoldEnhance EM; Nanoprobes) for 3 minutes at room temperature according to the manufacturer's instructions. After washing with distilled water, the cells were fixed in 1% OsO_4 containing 1,5% potassium ferrocyanide in PBS for 60 minutes at room temperature and washed with distilled water. The cells were dehydrated in a series of graded ethanol solutions and embedded in epoxy resin. After the epoxy resin hardened, the plastic coverslip was removed from it. Ultrathin sections were cut horizontally to the cell layer and double stained with uranyl acetate and lead citrate. Samples were analysed with an electron microscope. Serial sections were collected on pioloform-coated copper grids and samples analysed in a Philips CM100 electron microscope (Philips, Eindhoven, The Netherlands).

Western blot analysis | Human monocytes were stimulated with *A. fumigatus* conidia for the indicated time points at an MOI of 10:1. Where appropriate, cells were pre-incubated with DMSO or the indicated concentrations of inhibitors for 30 minutes prior to stimulation. Cells were washed once in PBS prior to lysis in 1% Nonidet P-40 containing RIPA buffer: 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0,25% sodium deoxycholate, 1 mM NaF, 1 mM Na_3VO_4, and 1 mM PMSF plus a mixture of protease inhibitors (Roche Molecular Biochemicals). Cell lysis was performed on ice for 20 minutes, and samples were centrifuged. After protein estimation of supernatants, addition of SDS sample buffer, and boiling, samples were separated on SDS-PAGE and transferred to polyvinylidene difluoride membranes. Western blotting was performed according to the instructions of the manufacturer using the following primary Abs: rabbit anti-LC3 (NB100-2220; Novus), mouse anti-actin (mAb 1501; Millipore), mouse anti-tubulin (T5168; Sigma Aldrich), rabbit anti-Syk (sc-1077), rabbit anti-phospho-Syk (Tyr525/526, 2710; Cell Signalling Technology), rabbit anti-phospho Src (Tyr416; 2101; Cell Signalling Technology), and goat anti-ATG5 (sc-8666; Santa Cruz Biotechnology). Secondary Abs used in Western blotting were purchased from Cell Signalling Technology (anti-rabbit HRP and anti-goat HRP) as well as Millipore (anti-mouse HRP). The blots were developed using chemiluminescence (ECL; Thermo Scientific).

Measurement of ROS production in human monocytes | ROS measurements were performed by means of a DCFH assay [26]. Stock solution of DCFH-DA was dissolved in DMSO to a final concentration of 100 mM. Human monocytes ($2x10^5$/well) were plated on 96-well round-bottom plates, incubated at 37°C for the indicated time (2 hour) with or without hydrocortisone, and accordingly stimulated for 1 hour with *A. fumigatus* spores in the presence of DCFH-DA added to a final concentration of 10 mM. After 30 minutes of exposure, the content of the wells were transferred to vials and the fluorescence of the cells from each well measured by flowcytometry. Cells were acquired on a FACS-Calibur (BD Biosciences) and analysed using FlowJo software (Tree Star).

Killing of *A. fumigatus* spores by THP-1 cells | THP-1 cells were plated onto 12-well plates and allowed to differentiate to macrophages in the presence of PMA (100 mg/mL). Cells that were adherent after 48 hour were used in phagocytosis and killing experiments. To measure macrophage

killing of conidia, PMA was removed by adding fresh media, and THP-1 cells were allowed to ingest *A. fumigatus* conidia at an MOI of 1:10 for 1 hour at 37°C. Medium containing nonadherent, nonphagocytosed conidia was removed, and wells were washed three times using warm PBS. Macrophages were then allowed to kill conidia for 2 and 6 hour before intracellular conidia were harvested. Macrophages were removed by scraping, placed in propylene tubes, snap frozen with the use of liquid nitrogen, and rapidly thawed at 37°C to lyse the THP-1 cells and harvest conidia. The process of cellular lysis was performed twice and confirmed by light microscopy. Lysates left overnight at 4°C in RPMI 1640. The percentage of killing (number of nongerminated spores per 100 counted conidia) in the culture well after 6-8 hour of incubation at 37°C was assessed under a microscope. Control wells containing only *A. fumigatus* conidia showed that the percentage of germination of the conidia used was always 90%.

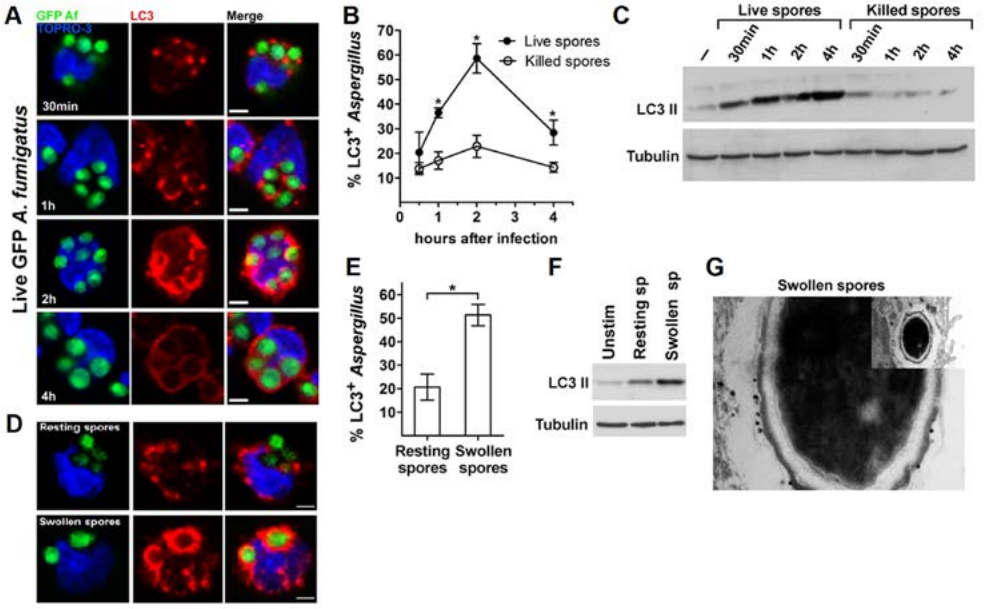

Figure 1 | LC3II is recruited to phagosomes of human monocytes during cell wall swelling of *A. fumigatus*
Monocytes (2x10^5 cells/condition) were infected with live (A, B) or PFA-killed (B) GFP *A. fumigatus* (GFP Af) at an MOI of 5:1. Cells were, stained for LC3II and the percentages of LC3-associated *A. fumigatus*-containing phagosomes (LC3$^+$ *Aspergillus* ; n=150/group) were quantified by measuring the number of LC3$^+$ *Aspergillus*-containing phagosomes out of the total number of engulfed *Aspergillus* spores, data are presented as mean ± SEM of three independent experiments. *p<0.0001, paired Student t test. (C) Monocytes (2x10^6 cells/condition) were infected with live or PFA-killed GFP *A. fumigatus* as in (A) and (B), and levels of LC3II and tubilin (as a loading control) protein were determined. (D and E) Monocytes were stimulated for 1 hour with PFA-killed dormant or swollen spores of GFP *A. fumigatus* as in (A). The percentages of LC3$^+$ *A. fumigatus*-containing phagosomes (LC3$^+$ *Aspergillus* ; n=150/group) were quantified, and data are presented as mean ± SEM of five independent experiments. *p<0.0001, paired Student t test. (F) Monocytes (2x10^6 cells/condition) were left untreated (Unstim) or stimulated with either PFA killed dormant (Resting sp) or swollen spores (Swollen sp) of *A. fumigatus*. Cell lysates were prepared, and LC3II and tubulin protein levels were determined. (G) Representative immunoelectron micrograph in which LC3II was labelled in monocytes stimulated for 1 hour with PFA-killed *A. fumigatus* swollen spores with 1.4-nm gold particles. Scale bars in (A) and (D) represent 5 µm.

Silencing of ATG5 expression by specific short interfering RNA | Short interfering RNA (siRNA) targeting was used to knockdown ATG5 expression in human THP-1 monocytes. A human monocyte nucleofector kit (Amaxa, Gaithenberg, MD) and Nucleofector device (Amaxa) were used for delivering siRNA into monocytes by following the instructions provided by the manufacturer. In brief, $1.5x10^6$ THP-1 cells were suspended in 100 mL human monocyte nucleofector solution (Amaxa) and transfected with siRNA at a final concentration of 100 nM using the V-001 program. Transfected cells were immediately diluted with prewarmed growth media and cultured in 12-well plates for 24 hour. THP-1 cells were allowed to differentiate for an additional 48 hours in the presence of PMA (25 mg/mL) and then used for experiments. The following siRNA pool of oligonucleotide sequences were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): ATG5 RNA interference (RNAi; sc-41445) and control RNAi (C RNAi) oligonucleotide sequences (sc-37003). Specific gene knockdowns were assessed by immunoblotting.

Statistical analysis | The data were expressed as means ± SEM. Statistical significance of differences was determined by Student t test and Bonferroni t test ($p<0.05$ was considered statistically significant). Analysis was done in GraphPad Prism software (version 5.0). All experiments were performed at least in triplicate and replicated at least twice.

Results

LC3II is recruited in *A. fumigatus* phagosomes upon fungal cell wall swelling

To evaluate whether autophagy proteins participate in immune responses against *A. fumigatus*, we monitored the kinetics of LC3II recruitment to phagosomes of primary human monocytes infected with live spores of GFP- or FITC-labelled *A. fumigatus* by immunostaining with the use of an anti-LC3 Ab. In contrast to the previously reported rapid $LC3^+$ phagosome formation, within minutes of the uptake of beads coated with TLR ligands [24], we noticed a delayed LC3II recruitment in *A. fumigatus*-containing phagosomes that was pronounced only after 2 hours of infection (Figure 1A). Subsequently, we investigated whether the formation of $LC3^+$ phagosomes is elicited by fungal molecules that are either released or exposed during intracellular fungal cell wall swelling of *A. fumigatus* spores [9]. Thus, we infected human monocytes with PFA-killed resting (dormant) or swollen *A. fumigatus* spores and assessed LC3II recruitment. Surprisingly, we noticed minimal LC3II recruitment in phagosomes even at late (4 hour) time points of infection of human monocytes with resting *A. fumigatus* spores (Figure 1B). Similarly, although monocyte infection with live *A. fumigatus* spores triggered high levels of LC3II protein expression, there was no evidence of significant LC3II protein expression in monocytes infected with PFA-killed *A. fumigatus* resting spores (Figure 1C). In contrast to resting *A. fumigatus* spores, swollen spores triggered robust $LC3^+$ phagosome formation (Figure 1D, 1E) and pronounced LC3II protein expression (Figure 1F). Collectively, these data reveal that LC3II protein recruitment in *A. fumigatus* phagosomes is not dependent on release of soluble factors and occurs upon fungal cell wall swelling.

In agreement with previous studies that reported lack of classic double membrane autophagosome formation in LC3$^+$ phagosomes containing TLR ligands [23], we found that *A. fumigatus* swollen spores were contained within single membrane phagosomes, which was also suggested by the presence of gold-conjugated Ab against LC3 only in the outer part of the phagosome membrane (Figure 1G).

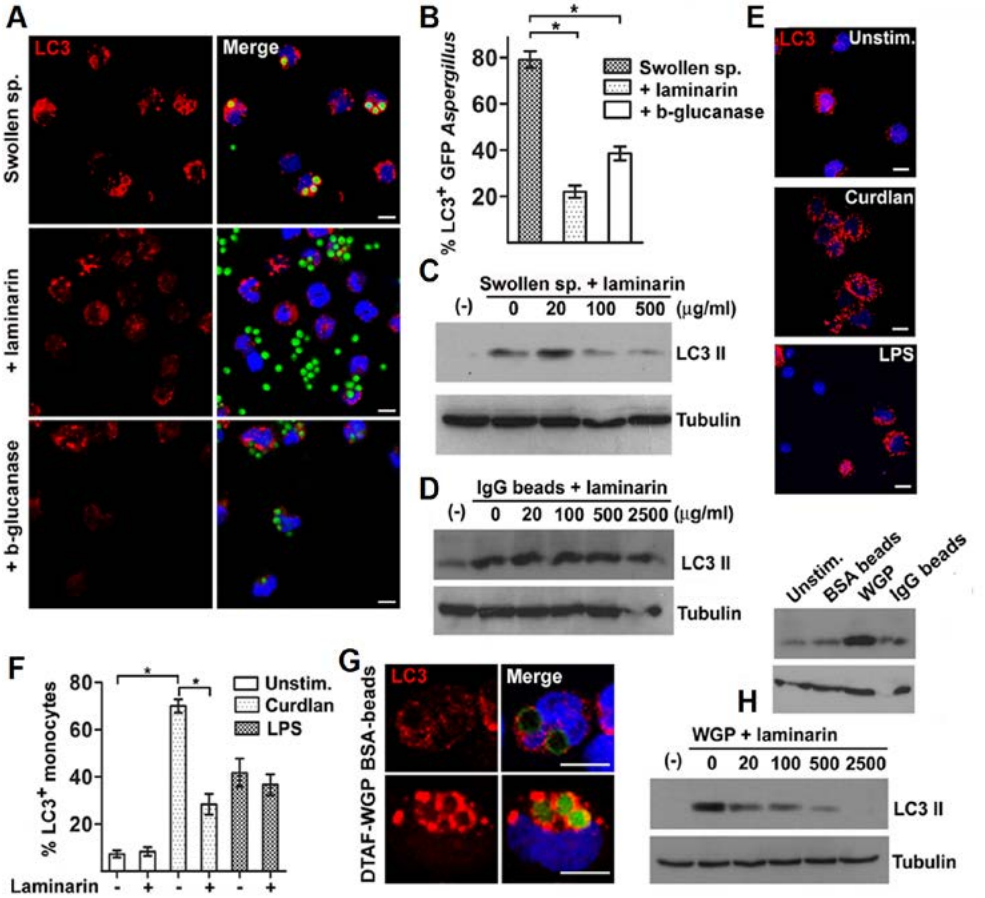

Figure 2 | β-glucan exposure in swollen *A. fumigatus* spores triggers LC3II recruitment in phagosomes
(A) Primary human monocytes (2x10^5 cells/condition) were infected with GFP *A. fumigatus* swollen spores with or without laminarin (500 mg/mL) or swollen spores (Swollen sp.) following overnight enzymatic digestion of β-glucan (β-glucanase) at an MOI of 5:1 for 1 hour. Cells were stained for LC3II, (B) the percentages of LC3$^+$ *A. fumigatus*-phagosomes (LC3$^+$ *Aspergillus*; n=150/group) were quantified, and data are presented as mean ± SEM of three independent experiments. *p<0.0001, paired Student t test. Monocytes (2x10^6 cells/condition) were stimulated with *A. fumigatus* swollen spores or in the presence of laminarin (C) or IgG coated 3 μm latex beads alone or in the presence of laminarin (D) for 1 hour. Protein levels of LC3II and tubulin (as a loading control) were determined in cell lysates. (E and F) Monocytes (2x10^5 cells/condition) were left untreated (Unstim.), stimulated with purified β-glucan (curdlan, 100 mg/mL) or LPS (100 ng/mL) with or without pretreatment with laminarin (500 mg/mL). The percentages of monocytes containing autophagosomes LC3 staining (LC3$^+$ monocytes; n=150/group) were quantified, and data are presented as mean ± SEM of two independent experiments. *p<0.0001, paired Student t test. (G) Monocytes (2x10^5 cells/condition) stimulated with FITC-labelled BSA beads or DTFA-labelled WGP at an MOI of 5:1 for 1 hour. (H) Monocytes (1x10^6 cells/condition) were left untreated (Unstim.), stimulated with BSA-coated beads, IgG-coated beads, or WGP with or without pretreatment with laminarin at an MOI of 10:1 for 1 hour. Protein levels of LC3II and tubulin were determined in the cell lysates. Scale bars in (A,E and G) represent 5 μm.

β-glucan exposure triggers LC3II recruitment in *A. fumigatus*-containing phagosomes

Recent studies demonstrated that resting *A. fumigatus* spores are immunologically inert because of concealing of pathogen associated molecular patterns by a surface layer of hydrophobin [27]. Importantly, swelling of *A. fumigatus* spores leads to surface exposure of the fungal polysaccharide β-1,3-D-glucan (β-glucan) and induction of robust inflammatory responses [28]. Therefore, we assessed whether stage-specific surface exposure of β-glucan in swollen *A. fumigatus* spores accounts for selective LC3II protein recruitment in *A. fumigatus* phagosomes. Accordingly, we performed enzymatic digestion of β-glucan in PFA-swollen *A. fumigatus* spores by using a β-1,3-D-glucanase and assessed the effect on LC3II protein recruitment in fungal phagosomes. Efficient digestion of β-glucan layer in *A. fumigatus* swollen spores was confirmed by immunofluorescence microscopy with the use of anti-β-glucan Ab. We found that enzymatic digestion of β-glucan resulted in significant reduction in $LC3^+$ *A. fumigatus* phagosome formation (Figure 2A, 2B) following infection of human monocytes with swollen *A. fumigatus* spores. Furthermore, laminarin, a non-immunostimulatory soluble β-glucan that acts as competitive inhibitor of β-glucan receptors [28], almost completely abolished $LC3^+$ *A. fumigatus* phagosome formation (Figure 2A, 2B) and LC3II protein induction in human monocytes stimulated with swollen *A. fumigatus* spores (Figure 2C). Notably, laminarin treatment had no effect in LC3II protein conversion in human monocytes stimulated with IgG-coated latex beads (Figure 2D).

The cell wall of *A. fumigatus* also contains Galactomannan moieties [29], and previous studies have implicated mannose- or mannan-specific receptors, including dendritic cell-specific ICAM-3-grabbing non-integrin and the long pentraxin PTX3, in the recognition of *A. fumigatus* [30,31]. To address the possible role of a mannose- or mannan-specific receptor in $LC3^+$ phagosome formation by swollen *A. fumigatus* spores, we pretreated human monocytes with *Saccharomyces cerevisiae*-derived mannan [31] prior to their addition to swollen spores and observed no effect on LC3II recruitment by immunofluorescence imaging or LC3II expression by Western blot analysis, in contrast to the effect of laminarin (Figure S1).

To confirm the ability of β-glucan to trigger $LC3^+$ phagosome formation, we stimulated human monocytes with different forms of purified insoluble β-glucan, including curdlan and yeast-derived WGP of ~3 μm size. Stimulation of human monocytes with curdlan particles elicited robust autophagosome formation that was blocked by pretreatment with laminarin (Figure 2E, 2F); in contrast, laminarin had no measurable effect in autophagy induction by LPS in human monocytes (Figure 2F). In addition, stimulation of human monocytes with fluorescein dichlorotriazine-labelled WGP resulted in a high degree of $LC3^+$ phagosome formation, comparable to that induced by stimulation with IgG-coated latex beads (Figure 2G). Similarly, we noticed high levels of LC3II conversion following stimulation of human monocytes with WGP, a response completely inhibited by laminarin (Figure 2H). Collectively, these studies demonstrate that β-glucan surface exposure in *A. fumigatus* fungal cell wall activates the recruitment of the autophagy protein LC3II in fungal phagosomes.

LC3II recruitment in *A. fumigatus* phagosomes depends on dectin-1/Syk signalling

Sensing of β-glucan by human myeloid cells predominantly occurs via engagement of the C-type lectin receptor dectin-1 [16,17]. Patients with the homozygous early stop-codon mutation Tyr238X in dectin-1 display lack of surface receptor expression, defective cytokine release, and hyper susceptibility to mucocutaneous fungal infections [20]. We tested whether dectin-1 is involved in β-glucan-induced $LC3^+$ phagosome formation by infecting monocytes of three patients having homozygous dectin-1 Tyr238X mutation (dectin-$1^{-/-}$) with PFA-killed resting and swollen *A. fumigatus* spores. We found that monocytes of dectin-$1^{-/-}$ patients had significant reduction in formation of $LC3^+$ phagosomes following infection with swollen *A. fumigatus* spores when compared with monocytes of dectin-$1^{+/+}$ controls (Figure 3A). In addition, blocking of dectin-1 in monocytes from healthy individuals with the use of a specific Ab resulted in significant reduction in $LC3^+$ phagosome formation following infection with swollen *A. fumigatus* spores (Figure 3B). Because TLR2 and TLR4 receptors are the main TLRs involved in sensing of *A. fumigatus* [2,14,15], we tested whether they also regulate autophagy protein recruitment in the phagosome. There was no evidence of significant reduction in LC3II recruitment in phagosomes containing swollen *A. fumigatus* spores following blockade of either TLR2 receptor using an anti-TLR2 Ab or TLR4 receptor using either an anti-TLR4 Ab or the TLR4 antagonist *B. quintana* LPS (Figure 3B). Because β-glucan has been reported to activate complement receptor 3 (CR3) in human phagocytes [17], we blocked this receptor by using competitive inhibition with N-acetyl-D-glucosamine [32,33] and assessed the effect in $LC3^+$ *A. fumigatus* phagosome formation. We did not find significant reduction in LC3II recruitment and LC3II protein conversion in human monocytes pre-exposed to N-acetyl-D-glucosamine and subsequently infected with swollen *A. fumigatus* spores (Figure S1). These studies suggest that LC3II recruitment in *A. fumigatus* phagosomes depends mainly on activation of dectin-1.

Coupling of Syk with dectin-1 and other CLRs activates multiple downstream pathways [16,17,34]. However, the role of Syk in phagosome maturation has not been earlier evaluated. In agreement with a stage-specific pattern of β-glucan exposure in the cell wall surface of *A. fumigatus*, we found selective activation of Syk following monocyte infection with swollen and not with resting *A. fumigatus* spores (Figure 3C). Importantly, treatment of human monocytes with two different Syk inhibitors almost completely abolished LC3 II recruitment in phagosomes containing swollen *A. fumigatus* spores and blocked LC3II protein conversion by Western blot analysis (Figure 3D, 3E). Similarly, treatment with Syk inhibitor blocked LC3 II recruitment in phagosomes containing purified β-glucan particles (WGP; Figure S2). Of interest, Syk inhibitors also blocked LC3II recruitment in phagosomes containing IgG-coated latex beads (Figure S2), implying that Syk controls $LC3^+$ phagosome formation upon activation of a broad range of PRRs that contain ITAM motifs.

Raf-1 kinase has been implicated in dectin-1 signalling via a Syk independent alternative noncanonical pathway of activation of NFκB [22]. Thus, we tested whether signalling mediated by raf-1 kinase is involved in LC3 recruitment in *A. fumigatus* phagosomes. Blocking of raf-1 kinase did not cause significant reduction in $LC3^+$ phagosome formation (Figure 3E, 3F) and LC3II protein expression (Figure 3G) in monocytes stimulated with swollen *Aspergillus* spores. Collectively, these studies demonstrate that dectin-1/Syk regulates the formation of $LC3^+$ *A. fumigatus* phagosomes.

Syk-dependent ROS production regulates formation of LC3$^+$ *Aspergillus*-phagosomes

Recent studies implicate NOX2-dependent ROS production in regulation of LC3II recruitment in phagosomes of murine macrophages containing TLR and FcγR ligands [24]. Since Syk regulates ROS production in response to β-glucan [16,17,34,35], we tested whether Syk-mediated LC3II recruitment in *A. fumigatus*-containing phagosomes was dependent on ROS production. We initially confirmed that similar to murine macrophages [35], treatment with Syk inhibitor in human monocytes resulted in complete inhibition of ROS production in human monocytes stimulated with swollen *A. fumigatus*

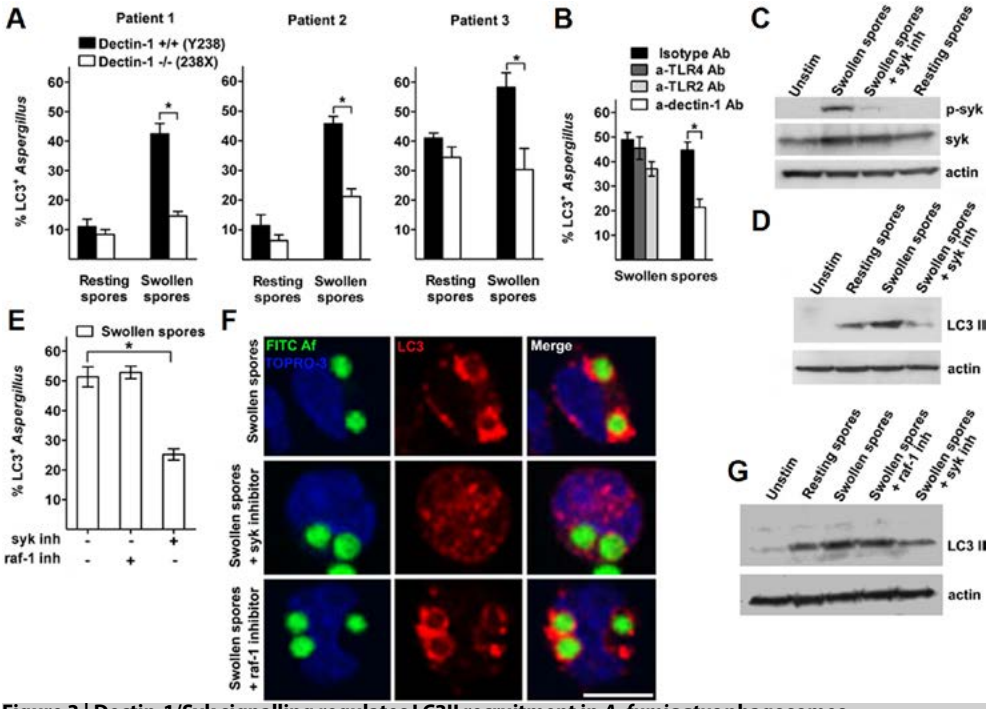

Figure 3 | Dectin-1/Syk signalling regulates LC3II recruitment in *A. fumigatus* phagosomes

(A) Monocytes (2x10^5 cells/ condition) isolated from homozygous patients with the stop-codon mutation 238X (dectin-1$^{-/-}$) and healthy controls (dectin-1$^{+/+}$) were infected with FITC-labelled resting or swollen *A. fumigatus* spores at an MOI of 5:1 for 1 hour at 37°C. Cells were stained for LC3II and the percentages of LC3$^+$ *A. fumigatus*-containing phagosomes (LC3$^+$ *Aspergillus* ; n=100/group) were quantified, data are presented as mean ± SD for each patient. *p<0.0001, paired Student t test. (B) Monocytes were stimulated with FITC-labelled *A. fumigatus* swollen spores following 30 minutes pre-incubation with blocking Abs for dectin-1 (10 mg/mL), TLR2 (10 mg/mL), or TLR4 (10 mg/mL) or the indicated isotype control Abs (10 mg/mL) at an MOI of 5:1 for 1 hour at 37°C. Cells were processed for immunofluorescence microscopy as in Fig. 1A. (C) Monocytes (2x10^6 cells/condition) were either left untreated (Unstim) or stimulated with resting or swollen *A. fumigatus* spores with or without 30 minutes pretreatment with Syk inhibitor (1 mM) at an MOI of 10:1 for 10 minutes at 37°C. Protein levels of phospho-Syk activity, total Syk and tubulin (as loading controls) were determined. (D) Monocytes (2x10^6 cells/condition) were stimulated as in (C) for 1 hour at 37°C, and levels of LC3II and tubulin were determined in cell lysates. (E and F) Monocytes (2x10^5 cells/condition) were stimulated with FITC-labelled swollen *A. fumigatus* spores with or without 30 minutes pretreatment with Syk inhibitor (574711; Calbiochem; 1 mM) or raf-1 inhibitor (GW5074; 40 mM) at an MOI of 5:1 for 1 hour and processed for immunostaining as in (A). The percentages of LC3$^+$ *A. fumigatus*-containing phagosomes (LC3$^+$ *Aspergillus* ; n=150/group) were quantified, and data are presented as mean ± SEM of four independent experiments. *p<0.0001, paired Student t test. Scale bar, 5 μm. (G) Monocytes (2x10^6 cells/condition) stimulated as in (E), and levels of LC3II and tubulin were determined in cell lysates.

spores (Figure 4A).

Importantly, patients with GCD have mutations in various components of NADPH oxidase and unique susceptibility to invasive *A. fumigatus* infection via incompletely characterized mechanisms [1,2,11]. Thus, we tested whether abolished ROS production in monocytes of CGD patients results in defective LC3II recruitment in *A. fumigatus*-containing phagosomes. When compared with monocytes of control healthy individuals, monocytes of three CGD patients displayed almost complete abolishment of $LC3^+$ phagosome formation following infection with *A. fumigatus* (Figure 4B, and 4C). In addition, we noticed decreased LC3II protein expression in lysates of monocytes from CGD patients infected with *A. fumigatus* in comparison with lysates of monocytes from healthy control patients infected with the fungus (Figure 4D). Therefore, NADPH-derived ROS production regulates LC3II recruitment in *A. fumigatus*-containing phagosomes, and this pathway is defective in patients with CGD.

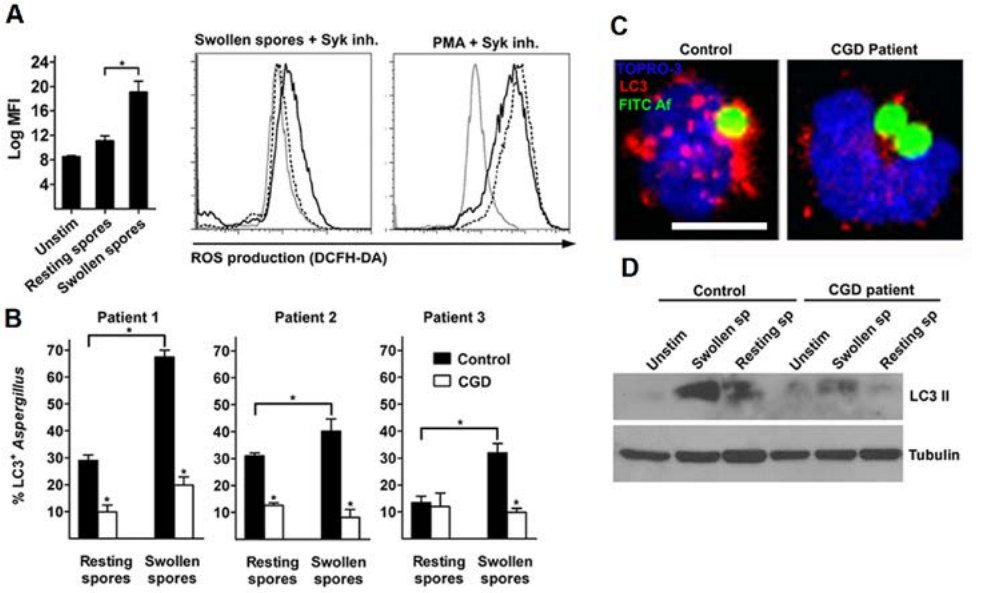

Figure 4 | Syk-dependent ROS regulates formation of $LC3^+$ *Aspergillus*-phagosomes.
(A) Primary human monocytes ($2x10^5$ cells/condition) were left unstimulated (Unstim) or infected with resting spores or swollen *A. fumigatus* spores at an MOI of 5:1 or stimulated with PMA (100 ng/mL) with or without 30-min pretreatment with Syk inhibitor (574711; Calbiochem; 1 mM). DCFH-DA was added during the last 30 minutes of stimulation, and intracellular ROS production was determined by measurement of mean fluorescence intensity (MFI). Data are presented as mean ± SEM from four independent experiments. Representative histograms from human monocytes left untreated (gray solid line), stimulated with either swollen *A. fumigatus* spores alone or PMA alone (black solid line), or in the presence of Syk inhibitor (black dashed line) are shown. *$p<0.005$, paired Student t test. (B and C) Monocytes ($2x10^5$ cells/condition) isolated from CGD patients and healthy controls were infected with FITC-labelled resting or swollen *A. fumigatus* spores at an MOI of 5:1 for 1 hour at 37°C. Cells were stained for LC3II and the percentages of $LC3^+$ *A. fumigatus*-containing phagosomes ($LC3^+$ *Aspergillus*; n=100/group) were quantified, and data are presented as mean ± SD for each patient. Representative immunofluorescence image of $LC3^+$ phagosomes containing FITC-labelled swollen *A. fumigatus* spores in monocytes obtained from healthy control and CGD patient. *$p<0.0001$, paired Student t test. (D) Monocytes ($1x10^6$ cells/condition) from a representative CGD patient and the corresponding healthy control were left untreated (Unstim) or stimulated with resting spores (Resting sp) or swollen spores (Swollen sp) of *A. fumigatus* at an MOI of 10:1 for 1 hour at 37°C, and levels of LC3II and tubulin were determined in cellular lysates.

ATG5 silencing attenuates phagosome maturation and killing of *A. fumigatus*

Recent studies demonstrated that silencing or knockdown of autophagy related genes ATG5 and ATG7 in murine macrophages resulted in impaired fusion of zymosan-containing phagosomes with lysosomes [2 3] and defective killing of *S. cerevisiae* [23] and *C. albicans* [36]. To evaluate the role of autophagy in human macrophage effector function against *A. fumigatus*, we performed silencing of ATG5 in THP-1-differentiated macrophages (Figure 5A), a human cell line previously shown to efficiently internalize and kill *A. fumigatus* [37]. Silencing of ATG5 in THP-1 macrophages resulted in significant reduction of the percentage of *A. fumigatus* spores within acidified lysosomes, as evidenced by LysoTracker staining (Figure 5B, 5C).

We next assessed the effect of ATG5 silencing in killing of *A. fumigatus* by THP-1 macrophages. Previous studies demonstrated that elimination of *A. fumigatus* occurs following an initial 2 hour lag phase and reaches maximum levels at approximately 6 hours of infection [9,10,37]. In agreement with previous studies [35], we found that THP-1 cells prevented germination of approximately 60%

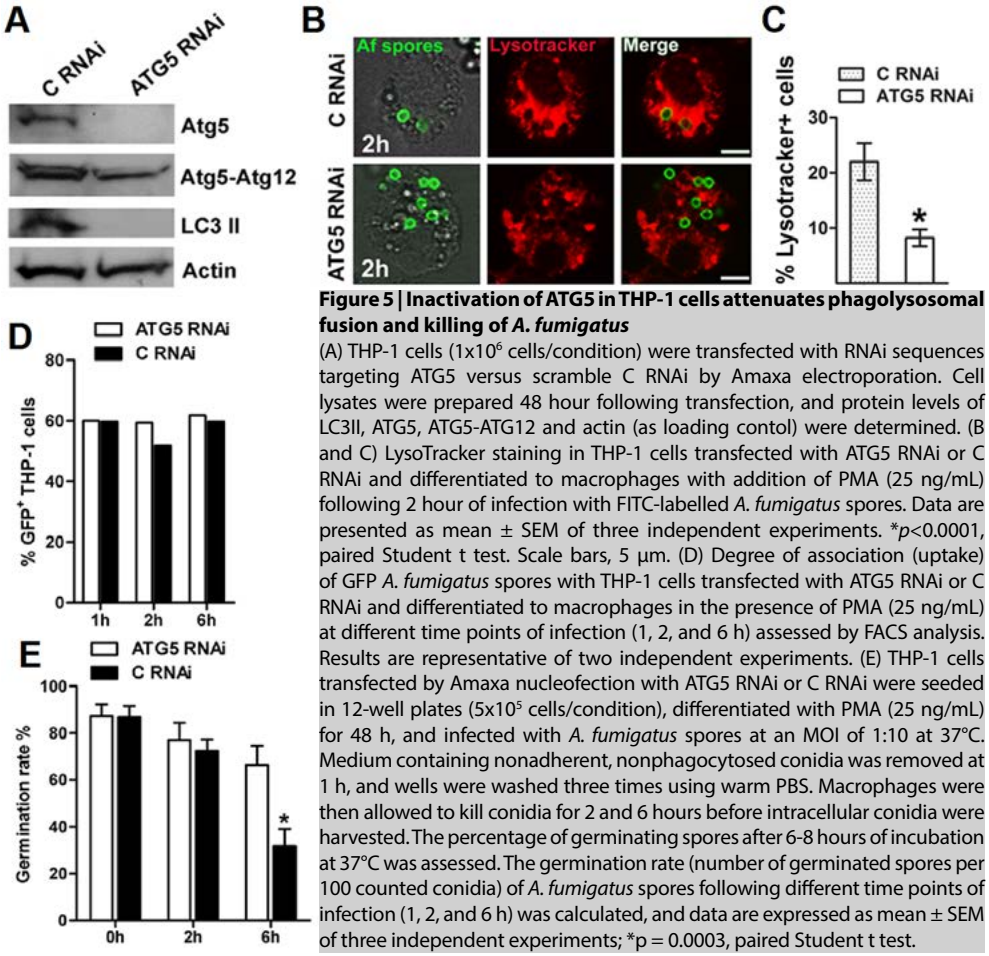

Figure 5 | Inactivation of ATG5 in THP-1 cells attenuates phagolysosomal fusion and killing of *A. fumigatus*

(A) THP-1 cells ($1x10^6$ cells/condition) were transfected with RNAi sequences targeting ATG5 versus scramble C RNAi by Amaxa electroporation. Cell lysates were prepared 48 hour following transfection, and protein levels of LC3II, ATG5, ATG5-ATG12 and actin (as loading contol) were determined. (B and C) LysoTracker staining in THP-1 cells transfected with ATG5 RNAi or C RNAi and differentiated to macrophages with addition of PMA (25 ng/mL) following 2 hour of infection with FITC-labelled *A. fumigatus* spores. Data are presented as mean ± SEM of three independent experiments. *$p<0.0001$, paired Student t test. Scale bars, 5 μm. (D) Degree of association (uptake) of GFP *A. fumigatus* spores with THP-1 cells transfected with ATG5 RNAi or C RNAi and differentiated to macrophages in the presence of PMA (25 ng/mL) at different time points of infection (1, 2, and 6 h) assessed by FACS analysis. Results are representative of two independent experiments. (E) THP-1 cells transfected by Amaxa nucleofection with ATG5 RNAi or C RNAi were seeded in 12-well plates ($5x10^5$ cells/condition), differentiated with PMA (25 ng/mL) for 48 h, and infected with *A. fumigatus* spores at an MOI of 1:10 at 37°C. Medium containing nonadherent, nonphagocytosed conidia was removed at 1 h, and wells were washed three times using warm PBS. Macrophages were then allowed to kill conidia for 2 and 6 hours before intracellular conidia were harvested. The percentage of germinating spores after 6-8 hours of incubation at 37°C was assessed. The germination rate (number of germinated spores per 100 counted conidia) of *A. fumigatus* spores following different time points of infection (1, 2, and 6 h) was calculated, and data are expressed as mean ± SEM of three independent experiments; *p = 0.0003, paired Student t test.

of *A. fumigatus* spores at 6 hours of infection, whereas there was little evidence of inhibition of *A. fumigatus* growth at earlier (2 hour) time points of infection (Figure 5E). Silencing of ATG5 in THP-1 human macrophages had no significant effect on the uptake of fungal spores (Figure 5D), but resulted in attenuated killing of *A. fumigatus* (Figure 5E). Collectively, these studies demonstrate that autophagy proteins regulate phagosome maturation and intracellular killing of *A. fumigatus*.

Corticosteroids block LC3II recruitment in *A. fumigatus*-containing phagosomes

Seminal studies in the 1970s demonstrated that corticosteroids block the fusion of lysosomes with *Aspergillus*-containing phagosomes in murine macrophages, leading to impaired killing of *A. fumigatus* [12,13]; however, a mechanistic explanation of the immunosuppressive action of corticosteroids on fungal phagosomes is lacking.

Since we found that components of autophagy regulate maturation of *A. fumigatus* phagosomes, we evaluated whether corticosteroids target this pathway. Therefore, we assessed $LC3^+$ phagosome formation in monocytes of patients with rheumatologic diseases before and 2 hours after i.v. administration of corticosteroids (Table I). Notably, we found a significant reduction in $LC3^+$ *A. fumigatus*-containing phagosomes following corticosteroid treatment in monocytes of all patients tested (Figure 6A, 6C). In addition, *ex vivo* administration of corticosteroids resulted in significant reduction in recruitment of LC3II protein in *A. fumigatus* phagosomes when compared with control untreated monocytes (Figure 6B).

Table 1 | Clinical and demographic characteristics of patients who received i.v. corticosteroids

No	Sex Age (y)	Underlying Disease	Disease Status	Comorbidities	Other Immunosuppressive Agents
1	F 62	RA	Active disease	Multiple sclerosis	Methotrexate, rituximab (anti-CD20)
2	F 68	RA	Active disease, rheumatoid lung	Hepatitis B	Leflunomide, rituximab (anti-CD20), receipt of anti-TNFα mAb in the past 12 mo
3	F 63	SLE	In remission	Cirrhosis (autoimmune hepatitis)	Azathioprine, hydroxychloroquine, rituximab (anti-CD20)
4	F 46	Polymyositis	In remission	Parkinson disease, pulmonary embolism	Methotrexate, low-dose prednisone (5 mg daily) in the past 6 mo
5	F 56	RA	Active disease, extra-articular manifestations (pericarditis)	NA	Methotrexate, rituximab (anti-CD20), receipt of anti-IL-6 mAb in the past 6 mo
6	F 61	RA	In remission	Chronic obstructive pulmonary disease	Methotrexate, low-dose prednisone (2.5 mg daily) in the past 6 mo

All patients received corticosteroids (methylprednisone or hydrocortisone) at a standard dose of 250 mg hydrocortisone equivalent as premedication for prevention of infusion reactions associated with the use of rituximab (anti-CD20 mAb). Blood was drawn before (0 h) and after (2 h) i.v. treatment with corticosteroids. F, Female; NA, not applicable; SLE, Systemic lupus erythematosus; RA, Rheumatoid arthritis.

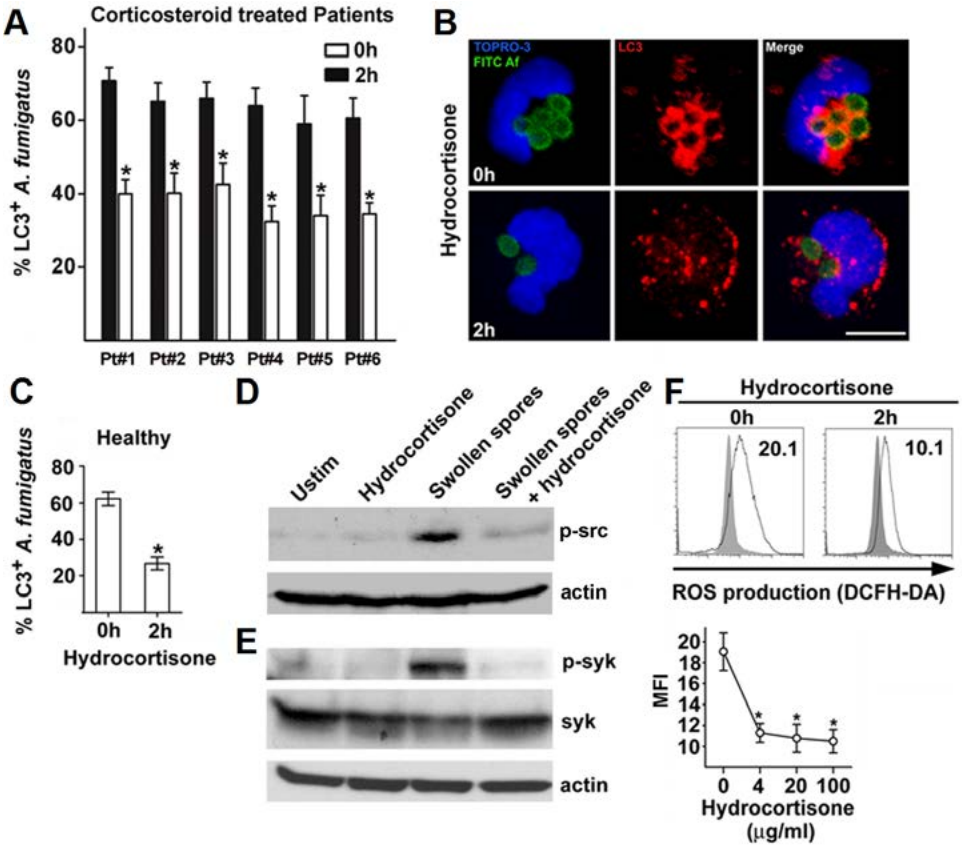

Figure 6 | Corticosteroids block LC3II recruitment in *A. fumigatus* phagosomes
(A) Monocytes (2x10^5 cells/condition) from six consecutive patients with rheumatologic diseases were collected before and 2 hour after i.v. administration of corticosteroids (250 mg hydrocortisone) and stimulated with swollen *A. fumigatus* spores at an MOI of 5:1 at 37°C. Cells were fixed, permeabilized, stained for LC3II and the percentages of LC3$^+$ *A. fumigatus*-containing phagosomes (LC3$^+$ *Aspergillus* ; n=150/group) before (0 h) and after (2 h) corticosteroid treatment, were quantified, and data are presented as mean ± SD for each patient. *p<0.05, paired Student t test. (B) Representative immunofluorescence image of LC3$^+$ phagosomes containing FITC-labelled swollen *A. fumigatus* spores (FITC Af) in monocytes obtained before (0 h) and after (2 h) administration of corticosteroids. Scale bar, 5 μm. (C) Monocytes (2x10^5 cells/condition) from healthy individuals (n=4) were stimulated before (0 h) and after (2 h) *ex vivo* exposure to hydrocortisone (20 mg/mL), fixed, and processed as in (A); data are presented as mean ± SEM of four independent experiments. *p<0.05, paired Student t test. Monocytes (2x10^6 cells/condition) from healthy individuals were either left untreated (Unstim) with or without 1 hour exposure to hydrocortisone (20 mg/mL) or stimulated with swollen *A. fumigatus* spores with or without 1 hour pre-exposure to hydrocortisone (20 mg/mL) at an MOI of 10:1 for 5 (D) or 10 minutes (E) at 37°C. Cell lysates were prepared, and levels of phospho-Src and phospho-Syk activity were determined, in the same lysates levels of tubulin and total Syk were determined as loading controls. (F) Monocytes (2x10^5 cells/condition) were left unstimulated or infected with swollen *A. fumigatus* spores at an MOI of 5:1 for 1 hour with or without pre-exposure (2 h) to increasing concentrations of hydrocortisone at 37°C. DCFH-DA was added during the last 30 minutes of stimulation, and intracellular ROS production was determined by measurement log mean fluorescence intensity (MFI). Representative histograms from monocytes left untreated (gray area) or stimulated with swollen *A. fumigatus* spores (black solid line) with or without pre-exposure to hydrocortisone (20 mg/mL) are shown. Differences in ROS production between experimental groups were quantified, and data are presented as mean ± SEM from four independent experiments. *p<0.0001, paired Student t test.

We subsequently assessed whether dectin-1/Syk signalling regulating antifungal autophagy responses is also targeted by corticosteroids. Of interest, we found no difference in the uptake of *A. fumigatus* spores and dectin-1 expression following administration of corticosteroids (Figure S3). Because corticosteroids block TCR signalling by inhibiting phosphorylation of ITAM motifs of TCR-mediated by tyrosine kinases [38,39], we reasoned that they might as well inhibit phosphorylation of Src and Syk tyrosine kinases in human monocytes. Importantly, we found that hydrocortisone administration caused a rapid block in phosphorylation of Src and Syk kinases within 5 and 10 minutes of *A. fumigatus* infection, respectively (Figure 6D, 6E).

Corticosteroids inhibit ROS production in murine macrophages following infection with *A. fumigatus* [10]. Because we found that ROS production in response to *A. fumigatus* infection in human monocytes is dependent on Syk signalling, we reasoned that corticosteroid-mediated blockade in Syk activation would result in defective ROS production. Indeed, human monocytes treated with corticosteroids displayed a significant reduction in the levels of ROS production following infection with *A. fumigatus* (Figure 6E). These studies demonstrate that corticosteroids target autophagy protein recruitment in *A. fumigatus* phagosomes via inhibiting Src/Syk-dependent ROS production and provide a potential mechanism for their direct immunosuppressive properties in phagosome maturation and killing of *Aspergillus* spp.

Discussion

In the present work, we shed light on the signalling regulating *A. fumigatus* phagosome maturation and uncover a potential mechanism for development of invasive fungal disease in patients with CGD and corticosteroid-induced immunosuppression. In particular, we found that activation of dectin-1/Syk/ROS signalling upon exposure of β-glucan in *A. fumigatus* spores triggers the recruitment of autophagy protein LC3II in fungal phagosomes, a response that is abolished in monocytes of patients with CGD. Furthermore, by silencing ATG5 in human phagocytes, we demonstrated that autophagy protein assembly is important for maturation of *A. fumigatus* phagosomes and fungal clearance. Very important from a clinical point of view, we also discovered that corticosteroids target the pathway of $LC3^{+}$ *A. fumigatus* phagosome formation by causing an early block in phosphorylation of Src and Syk kinases and downstream production of ROS.

Autophagy is a lysosomal degradation pathway that, among other immune-related actions, mediates clearance of intracellular pathogens via their engulfment upon escape to the cytosol [40]. Little is known about the role of autophagy pathway in immunity against extracellular pathogens, including fungi. Recent studies implicating autophagy proteins in regulation of maturation of phagosomes containing TLR ligands prompted us to study the physiologic relevance of this pathway in immunity against *A. fumigatus* [23,24]. Our initial experiments identified that fungal cell wall swelling is the trigger for LC3II recruitment in *A. fumigatus* phagosomes. Of interest, these studies provide a mechanistic explanation of previous observations by electron microscopy on the intracellular lifecycle of *A. fumigatus*, suggesting that fungal cell wall swelling is a prerequisite for efficient phagosome maturation and killing of *A. fumigatus* by murine macrophages [9].

Since β-glucan is selectively exposed at the surface of the fungal cell wall surface upon swelling of *A. fumigatus* spores [28], we tested whether this could be the trigger for LC3II recruitment in fungal phagosomes. By using different assays, including β-glucan enzymatic digestion, competitive inhibition with laminarin, and stimulation with purified β-glucan particles, we found that $LC3^+$ *A. fumigatus* phagosome formation was dependent on cell wall β-glucan. Previous studies in the murine RAW macrophage cell line using zymosan, a crude fungal cell wall extract rich in β-glucan, reported robust $LC3^+$ phagosome formation around zymosan particles mediated by TLR2 engagement [23,24]. However, because RAW macrophages express low levels of the β-glucan-sensing receptor dectin-1 [41] and because zymosan is a mixture of β-glucan and TLR ligands, it was difficult to dissect the contribution of β-glucan sensing in LC3II recruitment. Overall, our study identified β-glucan as the key molecule activating recruitment of autophagy proteins in fungal phagosomes.

In a following set of experiments, we tested whether $LC3^+$ *Aspergillus* phagosome formation was defective in monocytes of patients with the homozygous early stop-codon mutation Tyr238X in dectin-1 (dectin-$1^{-/-}$). Indeed, we found a significant reduction in recruitment of LC3 protein in monocytes of dectin-$1^{-/-}$ patients when compared with control dectin-$1^{+/+}$ monocytes infected with *A. fumigatus*. Similarly, blocking dectin-1 in monocytes of healthy individuals with the use of a specific Ab resulted in significant reduction in $LC3^+$ *A. fumigatus* phagosomes, whereas blocking TLR2 and TLR4 did not affect LC3 recruitment. Our findings corroborate a recent study reporting that in murine dendritic cells dectin-1 activation was required for LC3II recruitment in *C. albicans* phagosomes [42]. Importantly, dectin-$1^{-/-}$ patients are not at risk for IA in the absence of additional immunosuppression [20,21]. In our studies, we noticed residual LC3II recruitment in *A. fumigatus* phagosomes of dectin-$1^{-/-}$ patients, which is suggestive of redundancy in upstream innate receptors implicated in antifungal autophagy responses. Although blocking of other known fungal PRRs, including mannose, mannan receptors, and CR3, had no significant effect on $LC3^+$ *A. fumigatus* phagosome formation, we cannot preclude that cooperative activation of other CLRs (e.g., dectin-2, Mincle) may play an important role in LC3II recruitment in fungal phagosomes.

In addition, we assessed the role of Syk in LC3II recruitment in *A. fumigatus* phagosomes. Pharmacologic inhibition of Syk almost completely abolished LC3 protein recruitment in *Aspergillus* phagosomes. Notably, inhibition of raf-1 kinase that also activates an alternative signalling pathway downstream of dectin-1 had no impact on $LC3^+$ phagosome formation. Because Syk is downstream of many different signalling receptors [34], our finding could have broad spectrum implications on regulation of autophagy responses following sensing of endogenous or pathogen-related ligands. Importantly, a recent study in $Syk^{-/-}$ bone marrow chimeric mice found an indispensable role of Syk in intracellular killing of *A. fumigatus* by neutrophils and alveolar macrophages [43]. An important role of dectin-1/Syk signalling in acidification of phagosomes containing β-glucan-coated particles has also recently been reported [44]. In agreement, we found that inhibition of Syk impaired acidification, as evidenced by defective CD63 protein recruitment [45] in *A. fumigatus* phagosomes upon infection of primary monocytes and THP-1-differentiated macrophages (Figure S4).

NADPH oxidase-derived ROS production was recently shown to regulate recruitment of autophagy proteins in phagosomes of murine macrophages containing TLR or FcγR ligands [24]. In agreement with previous studies in murine and human phagocytes demonstrating that ROS production in response to zymosan is dependent on activation of Syk [35], we found that ROS production was selectively induced in response to swollen *A. fumigatus* spores in a Syk-dependent fashion. Studies in monocytes of CGD patients also revealed a block in $LC3^+$ *A. fumigatus* phagosome formation, confirming that NADPH-derived ROS also regulate recruitment of autophagy proteins in fungal phagosomes. Because patients with CGD have increased susceptibility to IA [1,2,11], and macrophages of mice with mutations in NADPH oxidase display defective phagolysosomal fusion and killing following the uptake of *A. fumigatus* spores [10], our studies suggest that defective autophagy protein recruitment could play an important role for development of invasive fungal infections in CGD.

Previous studies in murine macrophages demonstrated an important role of ATG7 and ATG5 proteins in phagosome maturation and clearance of yeast, including *S. cerevisiae* and *C. albicans* [23,37]. We also found that silencing of ATG5 in human THP-1 macrophages did not affect the uptake of fungal spores, but resulted in impaired maturation of *A. fumigatus* phagosomes and attenuated killing of the fungus. In humans, there are no previous studies to suggest a link between defective autophagy protein function and invasive fungal disease. Because full disruption of ATG5 is lethal in mice [46] and human patients with homozygous loss-of-function mutations in ATG5 have not been described, it has been difficult to assess the direct *in vivo* role of autophagy in *Aspergillus* immunity. Future studies in conditional ATG5 knockout mice should define the *in vivo* role of autophagy in *A. fumigatus* host defence and allow studying this pathway in neutrophils and other immune cell types with important role in antifungal immunity. An important future direction of research is represented by genetic association studies of polymorphisms in autophagy genes with susceptibility to fungal infection, studies that could validate the present *in vitro* data in a clinical setting.

Finally, we assessed whether corticosteroids, the major risk factor for development of IA, target autophagy protein recruitment in *A. fumigatus* phagosomes. Surprisingly, we found that administration of a relatively low dose of corticosteroids blocked LC3 recruitment in *A. fumigatus* phagosomes within 2 hours of exposure. Because of the rapid inhibition of $LC3^+$ *A. fumigatus* phagosome formation by hydrocortisone, we reasoned that this effect is mediated by nongenomic action of corticosteroids on dectin-1/Syk signalling. Notably, corticosteroids had no effect on *A. fumigatus* uptake and expression of dectin-1. Because corticosteroids have been shown to block tyrosine kinase phosphorylation within minutes of exposure in T-cells [39,40] and B-cells [47], we focused on their effects in phosphorylation of Src and Syk kinases in monocytes. Notably, we found that hydrocortisone almost completely inhibited phosphorylation of both Src and Syk kinases within minutes of exposure. Because Syk regulates ROS production in response to *A. fumigatus* infection, and corticosteroids have been shown to block ROS in macrophages during fungal infection[10], we tested whether hydrocortisone blocked ROS production in monocytes infected with *A. fumigatus*. Indeed, hydrocortisone caused a significant reduction in ROS production following infection with *A. fumigatus*. Of interest, recent studies on T-cells demonstrate that glucocorticoids induce

macroautophagy prior to the induction of apoptosis, because of their ability to inhibit Src kinases and downstream inositol 1,4,5-triphosphate-mediated calcium signalling [48]. We also found evidence of increased macroautophagy in monocytes pretreated with corticosteroids, an effect that precluded the assessment of blockade in $LC3^+$ phagosome formation by Western blot analysis. Thus, our studies reveal a selective property of corticosteroids to inhibit LC3II recruitment in fungal phagosomes, which is regarded as a specialized form of autophagy.

Collectively, our studies demonstrate an important physiologic role of autophagy pathway in restriction of intracellular growth of *A. fumigatus* within human phagocytes. Furthermore, our findings on defective antifungal autophagy as a result of impaired dectin- 1/Syk/ROS signalling could provide a mechanistic explanation for the defective phagocyte function in two distinct groups of patients with increased susceptibility for IA. Future studies are warranted to explore the therapeutic potential of autophagy induction in these patients and better define the *in vivo* role of autophagy in antifungal immunity.

Acknoledgements

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Supplementary material

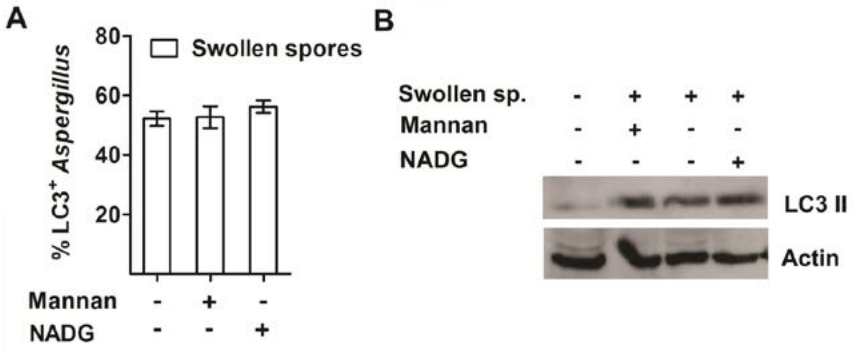


Figure S1 | Effect of N-acetyl-D-glucosamine (NADG) or yeast mannan pre exposure in LC3II recruitment in *A. fumigatus* phagosomes

(A) Monocytes (2x10^5 cells/condition) were infected with FITC-labeled *A. fumigatus* swollen spores with or without mannan (500 µg/mL) or NADG (500 µg/mL) at a MOI 5: 1 for 1h. Cells were stained for LC3II and the percentages of LC3$^+$ *A. fumigatus*-containing phagosomes (LC3$^+$ *Aspergillus*; n > 150 per group) were quantified and data are presented as mean ± SEM of 2 independent experiments. (B) Monocytes (2×10^6 cells/condition) were stimulated as in (A) and levels of LC3II protein were determined.

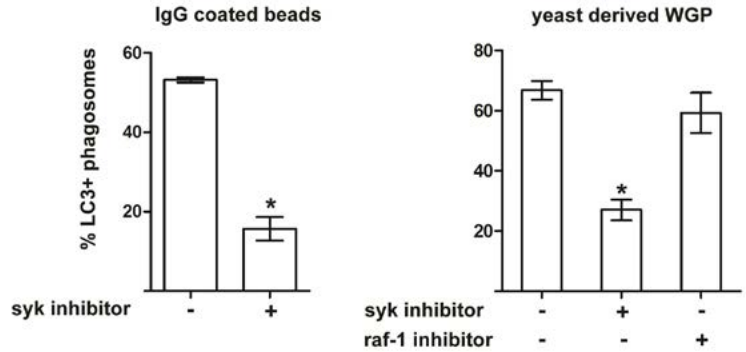


Figure S2 | Effect of Syk and raf-1 inhibitor in LC3II phagosome recruitment in monocytes stimulated with purified β-glucan particles (WGP) or IgG coated latex beads

Monocytes (2×10^5 cells/condition) were stimulated with IgG coated beads with or without pretreatment with a specific Syk inhibitor (574711, Calbiochem, 1 µM) or yeast derived whole glucan particles (WGP) both at MOI 5: 1 for 1h. Cells were stained for LC3II and the percentages of LC3$^+$ *A. fumigatus*-containing phagosomes (LC3$^+$ *Aspergillus*; n > 150 per group) were quantified and data are presented as mean ± SEM of 3 independent experiments. *, $P < 0.0001$, paired Student's *t* test.

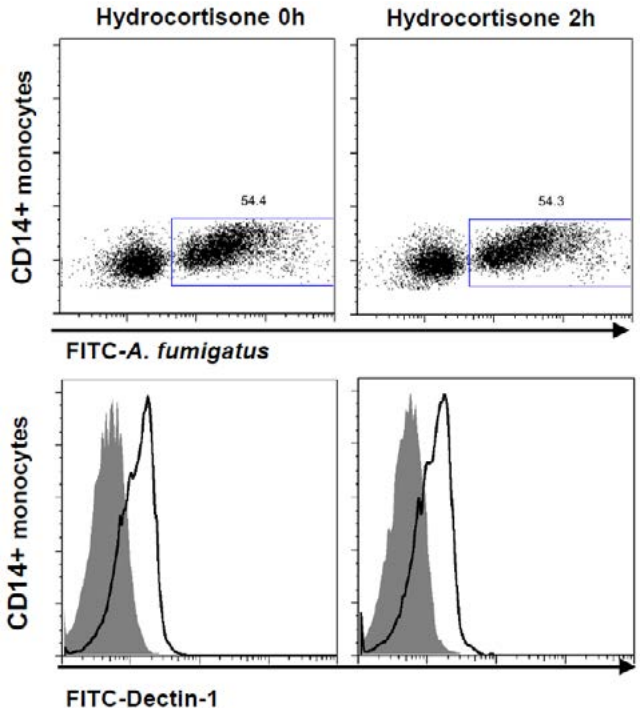


Figure S3 | Effect of hydrocortisone on dectin-1 expression and the uptake of FITC-labelled *A. fumigatus* spores by monocytesh

(A) Monocytes (2×10^5 cells/condition) from healthy individuals (n = 2) were stimulated before (0h) and after (2h) *ex vivo* exposure to hydrocortisone (20 µg/mL),infected with FITC-labelled swollen spores of *A. fumigatus* for 1h, washed three times with PBS to removed unbound spores, and the degree of internalization of FITC-labelled *A. fumigatus* spores with monocytes was immediately assessed by FACS analysis. (B) Monocytes stimulated as in A were stained with the use of a FITC-conjugated dectin-1 mAb or appropriate isotype control and levels of dectin-1 surface expression were analyzed by flowcytometry.

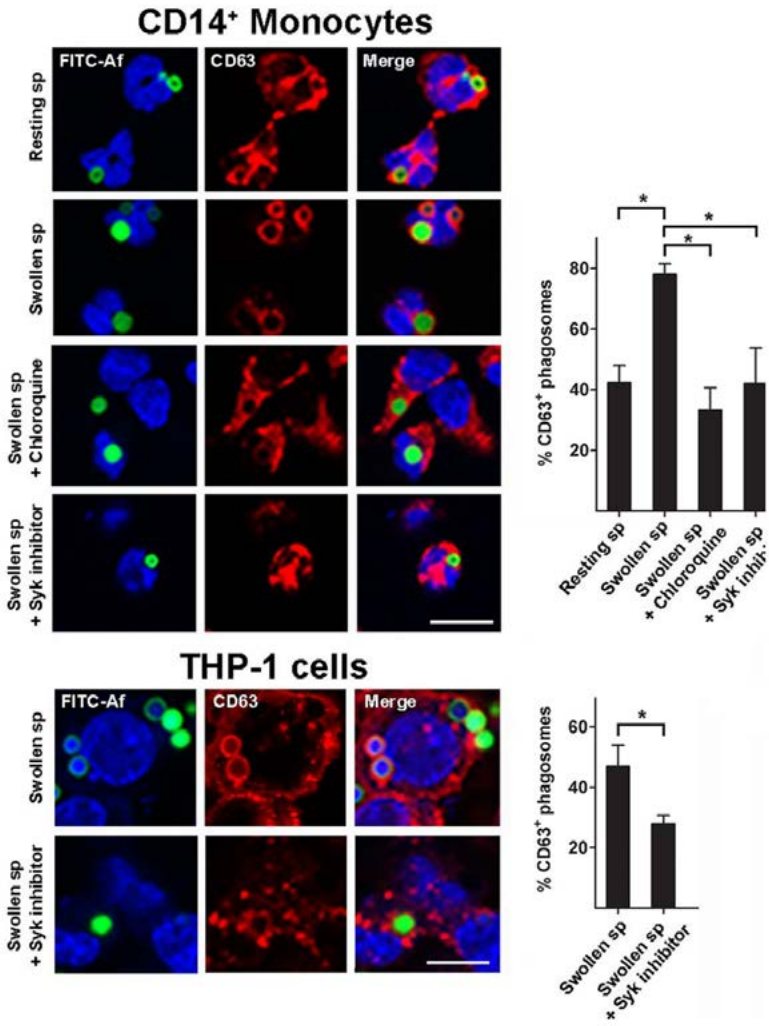
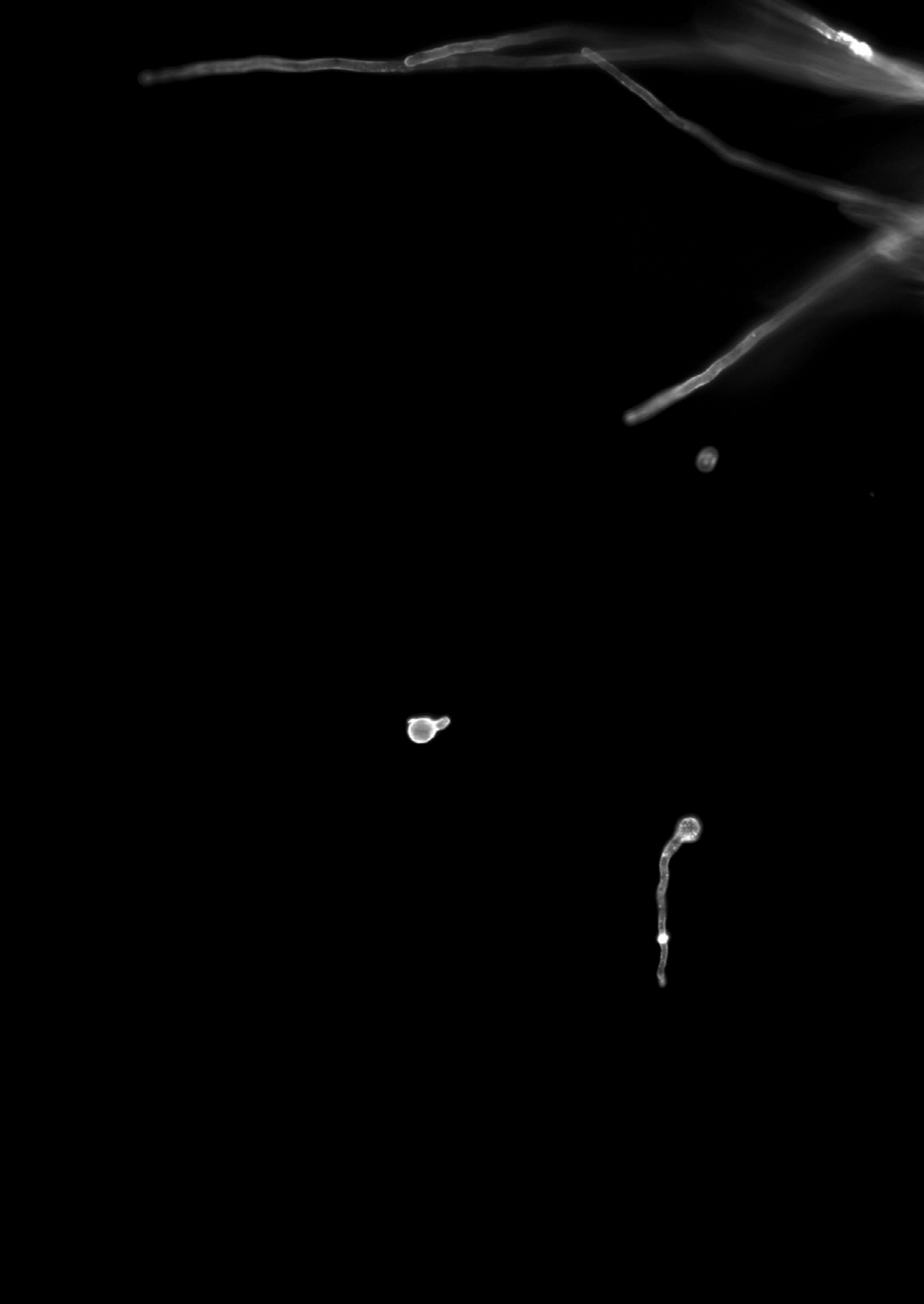


Figure S4 | Syk signalling regulates acidification of *A. fumigatus phagosomes*
Monocytes (2×10^5 cells/condition) isolated from healthy individuals and THP-1 PMA-differentiated macrophages (10^5 cells/condition) were stimulated with FITC-labelled resting or swollen spores of *A.fumigatus* with or without 30 min pretreatment with Chloroquine (Sigma, 100 μM) or a specific Syk inhibitor (574711, Calbiochem, 1 μM) at MOI 5: 1 for 1h. Cells were stained for CD63 and the percentages of $CD63^+$ *A. fumigatus*-containing phagosomes (> 150 per group) were quantified and data are presented as mean ± SEM of 3 independent experiments. *, $P < 0.0001$, paired Student's *t* test.



Chapter 4

Aspergillus fumigatus-induced interleukin-22 is not restricted to a specific T-helper subset and is dependent on complement receptor 3

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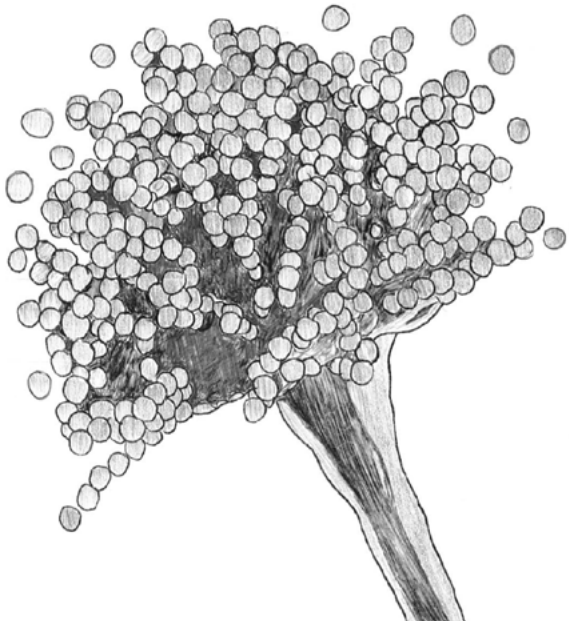

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Abstract

T-helper cell responses induced by *Aspergillus fumigatus* have been extensively investigated in mouse models. However, the requirements for differentiation and the characteristics of *A. fumigatus*-induced human T-helper (Th) cell subsets remain poorly defined. We demonstrate that *A. fumigatus* induces Th1 and Th17 subsets in human PBMCs. Moreover, we show that the cytokine IL-22 is not restricted to a specific T-helper subset, in contrast to IL-17A. The pattern recognition and cytokine pathways that skew these *Aspergillus*-induced T-helper responses are TLR4- and IL-1-, IL-23-, and TNFα- dependent. These pathways are of specific importance for production of the cytokines IL-17A and IL-22. Additionally, our data reveals that the dectin-1/ spleen tyrosine kinase (Syk) pathway is redundant, and TLR2 has an inhibitory effect on *Aspergillus*-induced IL-17A and IL-22 production. Notably, blocking complement receptor 3 (CR3) significantly reduced *Aspergillus*-induced Th1 and Th17 responses, and this was independent on the activation of the complement system. CR3 is a known receptor for β-1,3-glucan, however blocking CR3 had significant effects on T-helper responses induced by heat-killed *Aspergillus* conidia, which have minimal β-glucan expression on their cell surface. Collectively, these data characterize the human T-helper subsets induced by *Aspergillus*, demonstrate that the capability to produce IL-22 is not restricted to a specific T-cell subset, and provide evidence that CR3 might play a significant role in the adaptive host defence against *Aspergillus,* although the ligand and its action remains to be elucidated.

Introduction

The primary line of defence against *Aspergillus fumigatus* is mediated by neutrophils and other cells of the innate immune system [1]. In addition to the innate immune response, adaptive T-helper (Th) responses also play a crucial role during invasive aspergillosis (IA). The Th1 response is associated with a protection in IA [2]. However, conflicting data are reported regarding the role of Th17 responses in the host defence against *A. fumigatus.* A protective role for IL-17 is described by Werner *et al.* who showed that dectin-1$^{-/-}$ mice have decreased IL-17 production, and subsequently reduced *A. fumigatus* clearance [3,4]. In contrast, other studies show that IL-17 promotes inflammation and reduces resistance to the fungal infection [5,6]. Although these studies have investigated the role of IL-17 in mice, our group has previously reported that the human host response against *A. fumigatus* is mainly driven by the Th1 response, rather than the Th17 response [7]. In addition, human mononuclear cells have been shown to express a primarily Th1 biased cytokine profile in response to stimulation with *Aspergillus* [8], and when Th2 responses are being suppressed enhanced protective Th1 response develop in mice [9]. Furthermore, mice are resistant to *A. fumigatus* when proper IL-12 dependent Th1 responses are induced [9,10].

Even though various reports have focused on Th1 and Th17 responses against *A. fumigatus,* a role for the cytokine IL-22, which is a characteristic cytokine of the Th17 response, has not been addressed in human host responses. Recently, a protective role for IL-22 was demonstrated in the early host defence against *A. fumigatus* in a murine model of invasive pulmonary aspergillosis [11]. Furthermore, the induction of IL-22 contributes to lung pathology in a murine model of allergic bronchopulmonary aspergillosis (ABPA) [12]. In the host defence IL-22 is primarily responsible for the induction of anti-microbial peptides [13] and is mainly produced by CD4^{+} T-cells, Natural Killer (NK) cells and NKT-cells [13-19]. Within the CD4^{+} population, IL-22 is mainly produced by cells of the Th17 lineage [13], but also T-cells that are specialized in the production of IL-22 and TNFα; named Th22 cells [19].

Still, little is known about which cells mainly produce IL-17 (IL-17A), IFNγ and/or IL-22, and which recognition pathways and cytokines play a role in the induction of these cytokines in response to *A. fumigatus* in humans. In the present study we investigated the *A. fumigatus*-induced characteristic T-helper cytokines IL-17, IL-22, and IFNγ in PBMCs to elucidate which human cells primarily produce these cytokines, and which pattern recognition receptors and cytokines are involved in the induction of these cytokines in response to *A. fumigatus.*

Experimental procedures

Healthy volunteers and patients | Blood samples from healthy controls and patients were obtained after written informed consent. Three patients with homozygous Y238X mutations in exon 6 of *CLEC7A* gene (the gene encoding dectin-1) provided blood samples. In these patients diminished dectin-1 expression and failure to induce a cytokine response to β-glucan was demonstrated previously [20].

Aspergillus | A clinical isolate of *Aspergillus fumigatus* V05-27, which was previously characterized [21], was used for all stimulations. Conidia and hyphae were prepared and heat-inactivated (HI) as described previously [22]. A concentration of $1x10^7$/mL was used in the experiments.

β-1,3-glucan immunofluorescence of heat inactivated *Aspergillus* | To determine β-1,3-glucan expression after heat inactivation of *Aspergillus*. The HI conidia and hyphae were incubated for 30 minutes with mouse anti-β-1,3-glucan (Biosupplies, Bundoora, Australia). Subsequently the *Aspergillus* was washed and antibodies directed to β-glucan that were bound to *Aspergillus* were secondarily stained by goat anti-mouse alexa^{488} (Invivogen) according to the protocol supplied by the manufacturer. Immunofluorescence was observed at 400x magnification using a Zeiss Axio imager M1 fluorescence microscope, equipped with MRm camera (Carl Zeiss, Sliedrecht, The Netherlands).

PBMC isolation | Venous blood was drawn into 10 mL EDTA tubes, and PBMCs were isolated as described previously [21]. In brief, blood was diluted in phosphate buffered saline (PBS) (1:1) and fractions were separated by Ficoll (Ficoll-Paque Plus, GE healthcare, Zeist, The Netherlands) density gradient centrifugation. Cells were washed twice with PBS and resuspended in RPMI-1640 culture medium (Gibco, Invitrogen, Breda, The Netherlands) supplemented with 10 μg/mL gentamicin, 10mM L-glutamine and 10mM pyruvate (Gibco). The cells were counted using a particle counter (Beckmann Coulter, Woerden, The Netherlands) and the cell concentration was adjusted to $5x10^6$/mL.

CD4/CD56 cell depletion | To deplete the CD56 or CD4 cells from isolated PBMCs, cells were labelled using magnetic beads coated with anti-CD56 or anti-CD4 (MACS Miltenyi, Bergisch Gladbach, Germany). Subsequently, the cells were depleted over a depletion column according to the protocol supplied by the manufacturer. As control for the isolation procedure PBMCs were also runned over the columns without labelling with magnetic beads.

PBMC stimulation | PBMCs were plated in 96-well round-bottom plates (Corning, NY, USA) at a concentration of $2.5x10^6$/mL in a volume of 200 μL. They were either not stimulated or stimulated with $1x10^7$/mL HI conidia or hyphae for 24 hours or 7 days at 37°C and 5% CO_2. All stimulations were performed in medium containing 10% human serum, which was obtained from a serum pool of healthy volunteers.

Pattern recognition receptors were inhibited in PBMCs by pre-incubation for 1 hour with specific inhibitors. LPS derived from *Bartonella quitana* was used to block TLR4 at a final concentration of 20 ng/mL [23]. *B. quitana* LPS was extracted and purified as described previously [24]. Mouse anti-humanTLR2 (eBioscience, Halle-Zoersel, Belgium) and control mouse IgG1 (eBioscience), anti-human integrin β2 (αCR3) and control goat IgG (R&D systems Minneapolis, MN, USA) were used in a final concentration of 10 μg/mL. Laminarin was kindly provided by Professor David Williams of Tennessee University

and was used in a final concentration of 50 ng/mL to inhibit dectin-1. Spleen tyrosine kinase (Syk) kinase inhibitor was purchased from Calbiochem (Merck, Darmstadt, Germany) and was used in a concentration of 50 nM. In order to check the blockade of the PRRs, PBMCs were stimulated with the TLR4 ligand LPS (10 ng/mL) from *Escherichia coli* serotype O55:B5 (Sigma Chemical Co, St Louis, MO, USA); or with the TLR2 ligand Pam3Cys (1 μg/mL) (EMC microcollections, Tübingen, Germany). Inhibition of dectin-1 and Syk was validated by stimulation with HI *C. albicans* ($1x10^6$/mL). After 24 hours of stimulation at 37°C and 5% CO_2 IL-1β was measured by ELISA. All blockades resulted in a significant reduction of cytokine production (Figure S1A, B).

The cytokine pathways of IL-1, IL-23 and TNFα were investigated using supplementation of the cultures with recombinant human (rh) IL-23 (50 ng/mL) and rhTNFα (10 and 100 ng/mL) (R&D Systems). IL-1 receptor signalling was blocked by its natural receptor antagonist (Ra) IL-1Ra (10 μg/mL) (Amgen, Inc., Thousand Oaks, CA, USA), and IL-23 was blocked with mouse anti-human IL-23p19 (10 μg/mL) (R&D systems). sTNFRII (Enbrel) and human anti-human TNFα (Humira) were kindly provided by Dr. Renoud Marijnissen and Dr. Marije Koenders of the department of Rheumatology Radboud University Nijmegen Medical Centre the Netherlands, and were used to block TNFα in a final concentration of 100 μg/mL.

Cytokine measurements | IL-17A, IL-22, IFNγ, IL-1β and IL-23 were measured using commercially available ELISAs (R&D systems or eBioscience) according to the protocol supplied by the manufacturers.

Intracellular IL-17, IL-22, and IFNγ flowcytometry | Following 7 days stimulation, PBMCs were stimulated 4-6 hours with PMA (50 ng/mL) (Sigma-Aldrich), ionomycin (1 μg/mL) (Sigma-Aldrich) and Golgiplug (BD Biosciences, Breda, the Netherlands) according to the protocol supplied by the manufacturer. Cells were stained extracellular using PeCy7-conjugated anti-CD4 (BD Biosciences), PeCy7-conjugated anti-CD8 (Biolegend, San Diego, CA, USA) or PeCy7-conjugated anti-CD56 (Beckman Coulter) antibody. Subsequently the cells were fixed and permeabilized with Cytofix/Cytoperm solution (eBioscience) according to the protocol supplied by the manufacturer. Following permeabilization the cells were stained intracellular with $alexa^{647}$-conjugated anti-IL-17 (BD Biosciences), PE-conjugated anti-IL-22 (R&D systems) and FITC-conjugated anti-IFNγ (eBioscience) according to the protocol supplied by the manufacturers. The cells were measured on a FC500 flowcytometer (Beckman Coulter) and the data were analysed using CXP analysis software v2.2 (Beckman Coulter).

Statistical Analysis | Differences in IL-17, IL-22, and IFNγ production and the percentage of $CD4^+$ cells between the medium and *Aspergillus* stimulated samples were analysed with the Mann-Whitney-U test. Data of stimulations with and without inhibitors of PRRs, cytokines, or cytokine inhibitors were subjected to statistical analysis with the Wilcoxon signed rank test. A p-value of <0.05 was considered statistically significant, with $p<0.05$ = *, $p<0.01$ = **, and $p<0.001$ = ***. All experiments

were performed at least twice and data represent cumulative results of all experiments performed and are presented as mean ± standard error of the mean (SEM) unless indicated otherwise. Data were analysed using GraphPad Prism v5.0. The proportional Venn diagram was drawn using the eulerAPE application v2.0.3. [25,26].

Results

The pro-inflammatory adaptive cytokine response to *A. fumigatus*

We investigated the capacity of *A. fumigatus* conidia and hyphae to induce the cytokines IL-17A, IL-22 and IFNγ. Stimulation with conidia and hyphae induced a significant production of the Th1 cytokine IFNγ in human PBMCs, whereas IL-17A was induced in low amounts. Both conidia and hyphae also induced IL-22 in human PBMCs (Figure 1A).

To determine which cell populations expand after stimulation with *Aspergillus,* we performed flowcytometry analysis. We initially focused on $CD4^+$ cells, since T-helper cells are generally considered to be the main producers of IL-17A, IL-22 and IFNγ. PBMCs that were not stimulated with conidia showed relatively small populations of $IL\text{-}17A^+$ and $IL\text{-}22^+$ CD4 cells (1.2% SD 0.7 and 1.7% SD 0.9 respectively), whereas the $IFN\gamma^+$ CD4 cell population was 10.7% (SD 3.9). Stimulation with conidia induced a significant expansion of the $IL\text{-}17A^+$, $IL\text{-}22^+$ and $IFN\gamma^+$ CD4 T-cell population (Figure 1B). By gating on the *Aspergillus*-induced $IL\text{-}17A^+$, $IL\text{-}22^+$ and $IFN\gamma^+$ cells we observed that the majority of $IL\text{-}17A^+$ and $IL\text{-}22^+$ cells were $CD4^+$ cells. However, approximately half of the $IFN\gamma^+$ cells were negative for CD4 (Figure 1C). Subsequently, extracellular staining with the NK-cell marker CD56 and the cytotoxic T-cell marker CD8 revealed that a significant proportion of to the $IFN\gamma^+$ population was $CD56^+$ and $CD8^+$ (figure 1D). The remainder of the $IL\text{-}17A^+$ cells was positive for $CD8^+$ only. No $CD56^+$ $IL\text{-}17A^+$ cells were found (figure 1D). However, despite the fact that a fraction of the $IL\text{-}22^+$ cells was positive for CD8 or CD56, still approximately 9% of the $IL\text{-}22^+$ was negative for all the investigated surface markers (figure 1D). Interestingly, we found that the populations of $CD8^+$ and $CD56^+$ cells that were $IFN\gamma^+$ were already present in unstimulated PBMCs, and did not expand upon stimulation with *A. fumigatus* (figure 1E).

CD4 cells are the primary cellular source of *Aspergillus*-induced IL-17A, IL-22 and IFNγ

To demonstrate that $CD4^+$ cells are the major contributors to *Aspergillus*-induced IL-17A, IL-22 and IFNγ, we depleted $CD4^+$cells from PBMCs and compared this with normal PBMCs. Depletion of $CD4^+$ cells resulted in a complete loss of IL-17A production. Furthermore, IL-22 and IFNγ production were also significantly reduced, in most donors, to undetectable levels (figure 1F). Since several reports indicate that NK-cells can produce IL-22 [14,16-18], we also investigated the contribution of $CD56^+$ cells. Depletion of $CD56^+$ cells had no significant effect on IL-17A, IL-22 and IFNγ production (Figure 1G).

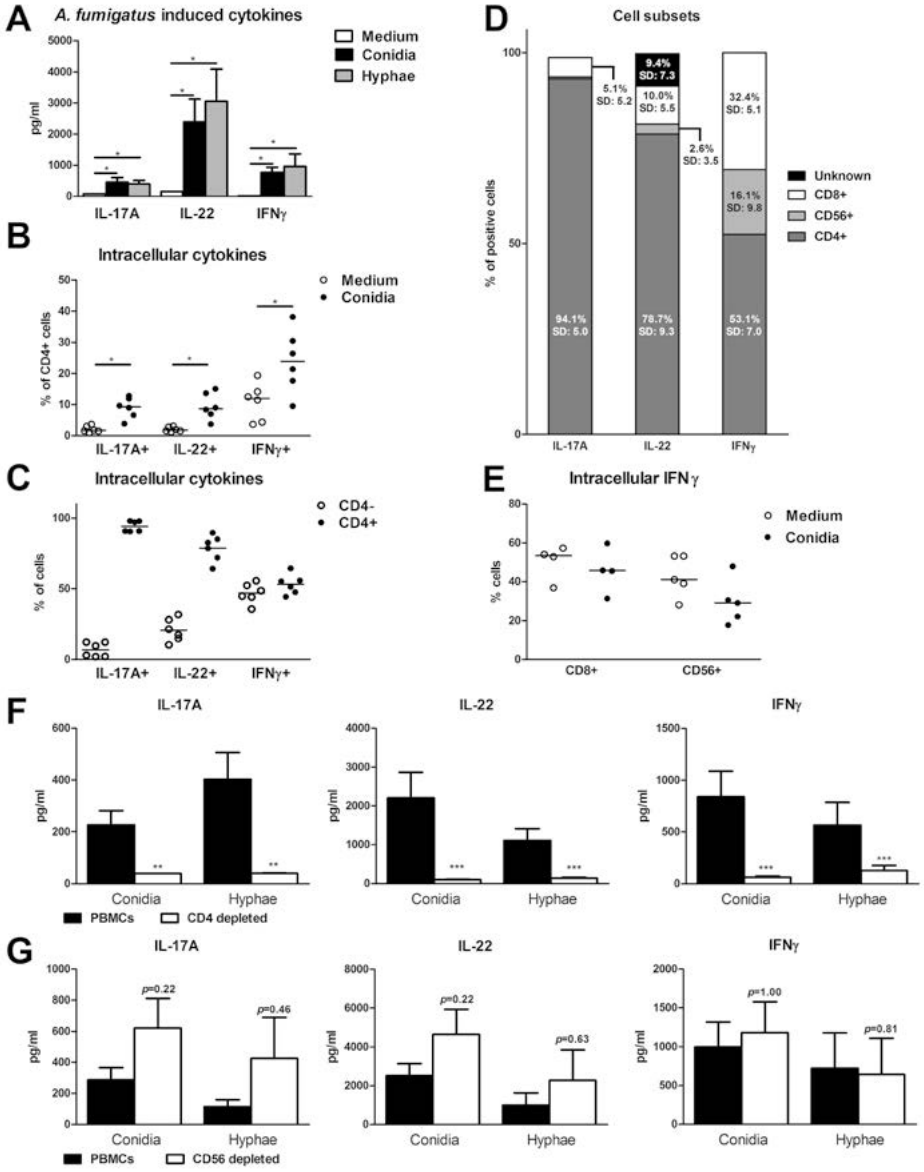

Figure 1 | Induction of IL-17A, IL-22, and IFNγ by *A. fumigatus,* and the cellular source of these cytokines
(A) IL-17A, IL-22 and IFNγ concentrations in culture supernatants of PBMCs (2.5x10^6/mL) (n=6 donors) that were stimulated with 10^7/mL HI *A. fumigatus* conidia or hyphae in the presence of 10% human serum. (B) Intracellular IL-17A, IL-22 and IFNγ in CD4 T-cells in the PBMCs of the experiments shown in panel A. (C) The percentage of CD4$^-$ and CD4$^+$ cells within the IL-17, IL-22 and IFNγ positive populations in *Aspergillus*-stimulated PBMCs of the experiments shown in panel a. (D) Assessment of surface markers CD56 and CD8 to elucidate the contribution of different cell types to the population of IL-17A$^+$, IL-22$^+$ and IFNγ$^+$ cells. (E) The expansion of CD8$^+$ IFNγ$^+$ and CD56$^+$ IFNγ$^+$ determined by comparing unstimulated to *A. fumigatus* stimulated PBMCs (n=4 for CD8 and n=5 for CD56). (F, G) IL-17A, IL-22 and IFNγ concentrations in culture supernatants of PBMCs, (F) PBMCs depleted of CD4$^+$ cells (n=10 donors for IL-17, n=12 donors for IL-22 and n=11 for IFNγ), (G) PBMCs depleted of CD56$^+$ cells (n=6 donors) that were stimulated with 10^7/mL HI *A. fumigatus* conidia or hyphae in the presence of 10% human serum. The Wilcoxon signed rank test was used to determine whether the means were significantly different.

IL-22 is not restricted to a specific T-helper subset

Since CD4$^+$ cells were the major population that were IL-17A$^+$, IL-22$^+$ and IFNγ$^+$ we investigated the phenotypic diversity of these CD4$^+$ T-cells by focusing on the expression of single or multiple cytokines. Stimulation with HI conidia resulted in the induction of IL-17/IL-22 and IL-22/IFNγ double positive CD4 cells (Figure 2A). Whereas we found a relatively small percentage of IL-17/IFNγ double positive CD4$^+$ T-cells. Strikingly, the cytokine IL-22 was not specifically expressed in a certain subset, but could be found in both the IL-17 and IFNγ positive T-cell populations. Moreover, A*spergillus* induces IL-17/IL-22/IFNγ triple positive CD4$^+$ T-cells (Figure 2A). To determine whether the IL-22$^+$ cells in our experiments match the phenotype of previously-described Th22 cells we assessed whether these cells co-expressed TNFα, a characteristic of Th22 cells [15,19,27]. Indeed, the majority of IL-22$^+$ cells co-expressed TNFα (Figure 2B). Additionally, we investigated how IL-1 and TNFα signalling can influence the IL-17 and IL-22 populations. Blocking IL-1 resulted in significantly decreased IL-17$^+$ and IL-22$^+$ populations and a trend towards decreased IL-17/IL-22$^+$ cells, whereas blocking TNFα with sTNFRII only significantly decreased the number of IL-22$^+$ cells (Figure 2C).

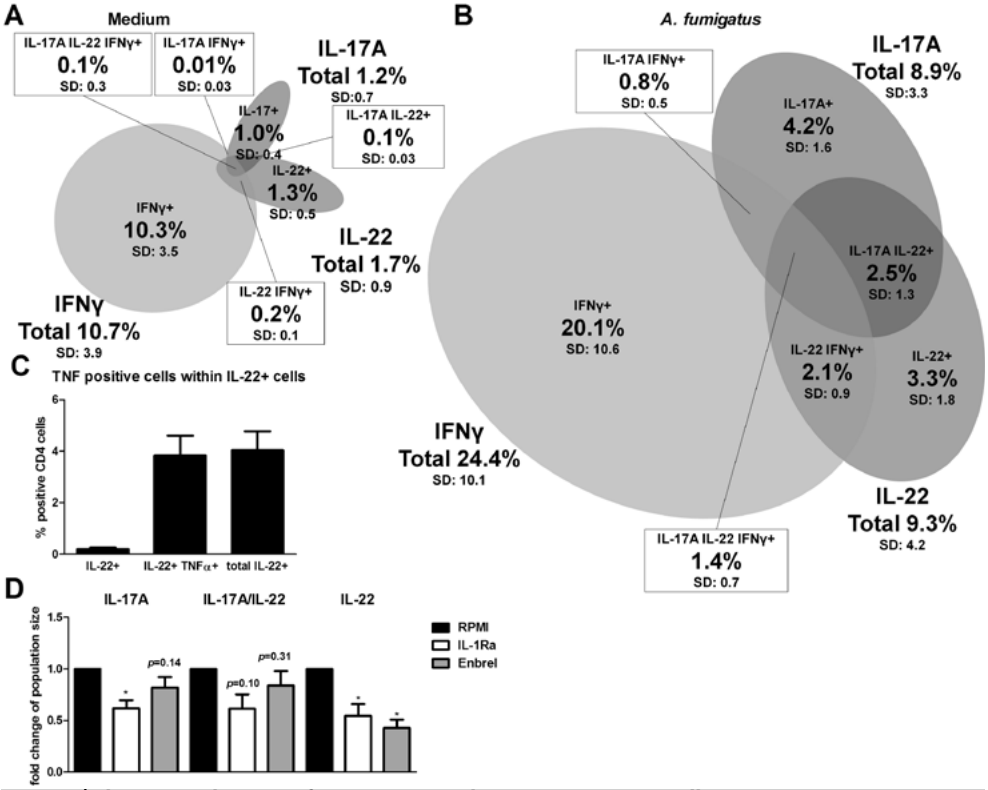

Figure 2 | Phenotypic diversity of IL-17A, IL-22 and IFNγ positive CD4 T-cells

Proportional Venn diagrams of IL-17A, IL-22, and IFNγ positive CD4 cell populations of (A) medium- and (B) *Aspergillus* conidia-stimulated PBMCs. Overlap between the ellipses represent double and triple positive CD4 cells. (C) Expression of TNFα within the IL-22$^+$ cell population of *Aspergillus*-stimulated PBMCs. (D) IL-17$^+$, IL-17/IL-22$^+$ and IL-22$^+$ CD4 cell populations of *Aspergillus* conidia- stimulated PBMCs (n=6 donors) in the presence or absence of IL-1Ra or sTNFRII (Enbrel) (Data represented as fold change from stimulation in absence of inhibitor).

Differential roles for TLR2 and TLR4 in *Aspergillus*-induced T-cell responses

We investigated the role of the Toll-like receptors in the induction of the IL-17, IL-22, and IFNγ responses to *A. fumigatus,* as TLR2 and TLR4 have been associated with the recognition of *A. fumigatus* [28-32]. PBMCs were pre-incubated with *B. quintana* LPS to block TLR4, and afterwards stimulated with conidia or hyphae. Pre-incubation with *B. quintana* LPS alone did not result in any cytokine induction in PBMCs (Figure S1C). Blockade of TLR4 resulted in a significantly reduced IL-22 production in conidia-stimulated PBMCs and reduced IL-17 and IL-22 production in hyphae-stimulated PBMCs, whereas IFNγ production was not affected by TLR4 blockade (Figure 3A). Blocking TLR2 resulted in an upregulation of IL-17 and IL-22 production by conidia stimulated PBMCs and a trend towards upregulation of IL-22 in hyphae-stimulated PBMCs (Figure 3B).

Redundant role for dectin-1/Syk signalling in *Aspergillus*-induced T-cell responses

Dectin-1 has been associated with the defence against *Aspergillus* in numerous reports [3,4,33-38]. Gessner and co-workers described that dectin-1 deficient mice lack the ability to induce IL-22 early in infection, which causes a decreased ability to induce antimicrobial peptides, resulting in an increased fungal burden and mortality [11]. To see whether the dectin-1/Syk signalling pathway is involved in the induction of IL-22 production by human T-cells, PBMCs were stimulated with conidia or hyphae in the presence of laminarin, a dectin-1 inhibitor. Blocking dectin-1 with laminarin did not result in any significant effect on hyphae or conidia-induced IL-17A, IL-22, or IFNγ (Figure 4A).

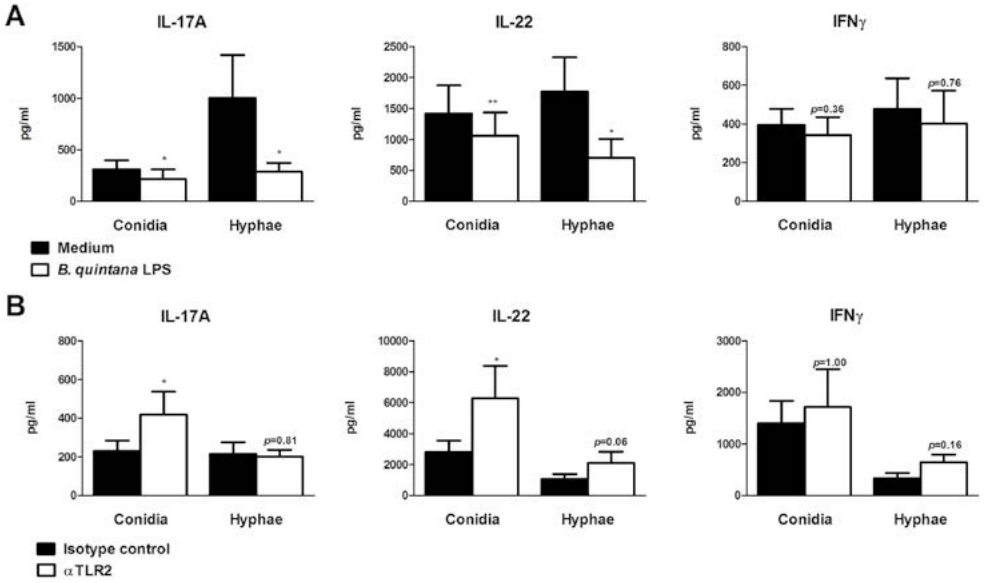

Figure 3 | Differential roles for TLR4 and TLR2 in *Aspergillus* induced T-cell responses

(A) IL-17, IL-22 and IFNγ were measured in culture supernatants of PBMCs ($2.5x10^6$/mL) stimulated with 10^7/mL *A. fumigatus* conidia (n=7 for IL-17, n=8 for IL-22, and N=9 for IFNγ) or hyphae (n=7 for IL-17, n=7 for IL-22, and n=11for IFNγ) for 7 days, that were pre-incubated with *B. quintana* LPS to block TLR4 and were compared to pre-incubation with culture medium. (B) Similarly, TLR2 was blocked by 1 hour pre-incubation using mouse anti-human TLRf2 monoclonal antibody which was compared to the isotype control (n=6).

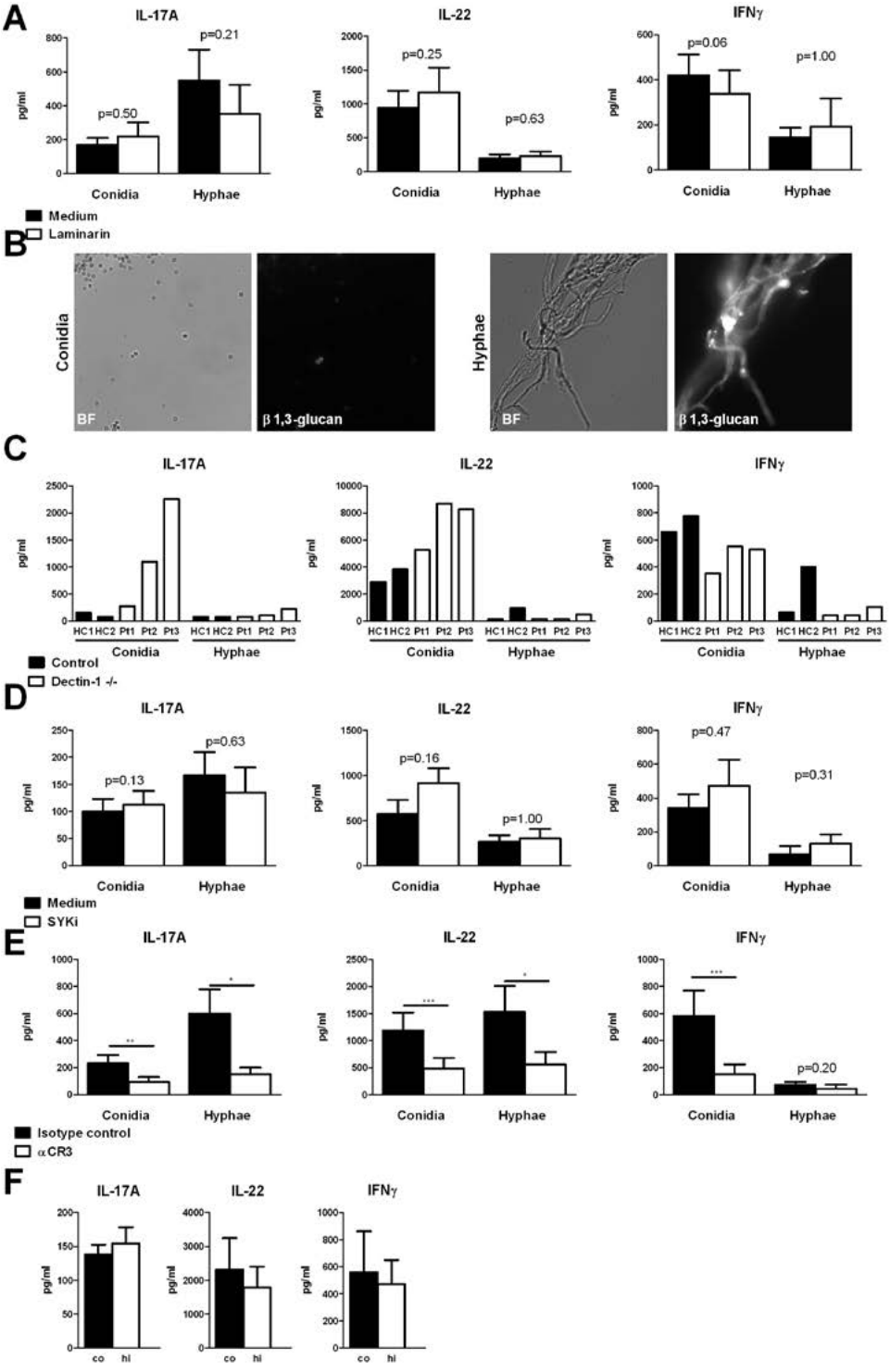
A
IL-17A
IL-22
IFNγ
pg/ml
p=0.50
p=0.21
p=0.25
p=0.63
p=0.06
p=1.00
Conidia
Hyphae
Medium
Laminarin
B
Conidia
Hyphae
BF
β 1,3-glucan
C
IL-17A
IL-22
IFNγ
HC1 HC2 Pt1 Pt2 Pt3
Control
Dectin-1 -/-
D
IL-17A
IL-22
IFNγ
p=0.13
p=0.63
p=0.16
p=1.00
p=0.47
p=0.31
Medium
SYKi
E
IL-17A
IL-22
IFNγ
p=0.20
Isotype control
αCR3
F
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Figure 4 | Redundant role of dectin-1 and Syk signalling in *Aspergillus*-induced IL-17A, IL-22, and IFNγ
(A) IL-17A, IL-22 and IFNγ in culture supernatants of PBMCs ($2.5x10^6$/mL) with 10^7/mL *A. fumigatus* conidia or hyphae for 7 days, that were pre-incubated with laminarin to block dectin-1, (n=8 for conidia and n=5 for hyphae). (B) Bright field (BF) and Immunofluorescence images after staining with mouse anti-β-1,3-glucan conjugated with goat anti-mouse IgG alexa^{488}. HI *Aspergillus* conidia (left panels) and HI *Aspergillus* hyphae (right panels) at 400x magnification and an exposure time of 900ms for fluorescence pictures. (C) IL-17A, IL-22 and IFNγ in culture supernatants of PBMCs ($2.5x10^6$/mL) from three patients with a homozygous dectin-1 Y238X polymorphism and two healthy controls without the polymorphism were stimulated with *A. fumigatus* conidia or hyphae for 7 days and IL-17, IL-22, and IFNγ were measured in the culture supernatant. IL-17, IL-22 and IFNγ in culture supernatants of PBMCs ($2.5x10^6$/mL) stimulated with 10^7/mL *A. fumigatus* conidia or hyphae in presence or absence of (D) Syk inhibitor (n=11 for conidia and n=6 for hyphae), or (E) goat anti-CR3 monoclonal antibody or an isotype control (conidia n=11 or hyphae n=6 for IL-17 and IL-22, n=8 for IFNγ). (F) IL-17, IL-22 and IFNγ in culture supernatants of PBMCs ($2.5x10^6$/mL) (n=6 donors) with 10^7/mL *A. fumigatus* conidia for 7 days in the presence of 10% human serum or 10% human serum that was heat inactivated for 30 minutes at 56°C. Differences of the means were analysed for significance using the Wilcoxon Signed Rank test.

However, there was one donor with unusual high IL-17 production in response to hyphae, which we excluded from analysis. It should be noted that the IL-17 production in this donor was almost completely blocked with laminarin.

We investigated whether *Aspergillus* conidia and hyphae expressed β-1,3-glucan on the surface by immunofluorescence staining. We found that β-1,3-glucan was only minimally present on a few conidia, which is in line with reports demonstrating that resting conidia do not express β-glucan [34]. In contrast, hyphae abundantly exposed beta-glucan (figure 4B). To confirm that dectin-1/Syk signalling does not play a significant role in the induction of IL-17A, IL-22 or IFNγ by *Aspergillus,* PBMCs of three patients with the homozygous mutation Y238X in dectin-1 (lacking dectin-1 expression on the membrane of the cell [20]) were stimulated with conidia and hyphae. PBMCs from these patients demonstrated either a similar or higher cytokine response to *Aspergillus* compared to PBMCs from a healthy control (Figure 4C).

Syk is the downstream signalling kinase of dectin-1 and other C-type lectin receptors (CLRs) [34]. Although, hyphae-induced IL-17 production tended to decrease upon blocking of dectin-1, inhibition of Syk demonstrated no difference in IL-17A, IL-22, or IFNγ production by *Aspergillus* stimulated PBMCs (Figure 4D).

The role for complement receptor 3 (CR3) for *Aspergillus*-induced T-cell responses

Recently, CR3 was demonstrated to recognize β-glucan, which leads to downstream signalling and activation of granulocytes [39]. Moreover, CR3 has also been associated with phagocytosis of pentraxin3 opsonised *Aspergillus* [40]. We investigated whether CR3 was involved in the induction of IL-17A, IL-22, and IFNγ by *A. fumigatus*. Blockade of CR3 resulted in a significant reduction of IL-17A, IL-22 and IFNγ responses induced by conidia. In response to hyphal stimulation, only IL-17A and IL-22 were significantly inhibited by blocking of CR3 (Figure 4E). Since the primary role of CR3 is recognition of C3 opsonised structures, we investigated whether active serum complement was required for *Aspergillus*-induced IL-17A, IL-22 and IFNγ. When human serum was heat-inactivated prior to stimulation, no differences in IL-17A, IL-22 and IFNγ induction were observed (Figure 4F).

The role of IL-1 and IL-23 in *A. fumigatus*-induced IL-17 and IL-22

The predominant cytokine produced by human PBMCs in response to *Aspergillus* was IL-22, while the IL-17A production was relatively low (Figure 1A). To elucidate the reason for the low IL-17A release by *Aspergillus* stimulated PBMCs we focused on the cytokines that regulate the Th17 response. Both IL-1β and IL-23 have been associated with the induction and maintenance of the Th17 response in humans [41]. Additionally, a critical role for IL-23 in the induction of IL-22 was observed in the early response against *A. fumigatus* [11]. Interestingly, IL-23 induction by PBMCs stimulated with conidia and hyphae was below the detection limit of the ELISA, while IL-1β production was detectable (Figure 5A). To investigate whether the absence of IL-23 production is responsible for the low production of IL-17 in response to *Aspergillus*, IL-23 was blocked with an anti-human IL-23p19 antibody. Compared to stimulation with the isotype control, IL-17A production was reduced to undetectable levels although this was not statistically significant, while IL-22 production was not affected (Figure 5B). Addition of IL-23 significantly increased IL-17A production but did not increase IL-22 production (Figure 5C). Blocking IL-1 signalling with IL-1 receptor antagonist (IL-1Ra) reduced IL-17A and IL-22 induction significantly (Figure 5D). These results suggest that IL-17A production requires both IL-1β and IL-23, whereas IL-22 production requires only IL-1β.

Role of TNFα in *A. fumigatus*-induced IL-17 and IL-22

Although IL-1β and IL-23 play key roles in the induction of T-helper responses by *A. fumigatus*, it cannot be excluded that other cytokines also play a role in the induction of the cytokines IL-17 and IL-22. To investigate the role of TNFα in *A. fumigatus*-induced IL-17 and IL-22 we stimulated PBMCs with HI conidia in the presence of 10 or 100 ng/mL TNFα. Both IL-17 and IL-22 production were dose dependently increased in the presence of TNFα when compared to stimulation with *Aspergillus* alone (Figure 6A). To further investigate the role of TNFα we stimulated PBMCs with HI conidia in the presence of sTNFRII (Enbrel) or human anti human TNFα (Humira), two drugs that are used to block TNFα signalling in IL-17 related diseases like rheumatoid arthritis (RA) [42]. Both blockers significantly reduced the IL-17 and IL-22 response (Figure 6B).

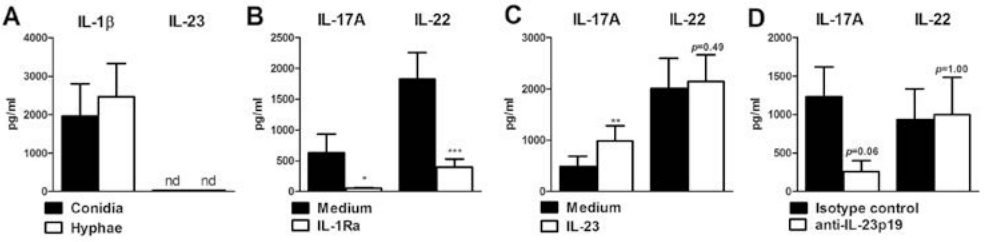

Figure 5 | Role of IL-23 and IL-1 in *A. fumigatus*-induced IL-17A and IL-22

IL-1β and IL-23 concentrations in PBMCs (2.5x10^6/mL) (n=7) that were stimulated with HI *A. fumigatus* conidia or hyphae 37°C and 5% CO_2 for 24 hours (A). IL-17 and IL-22 were measured in the culture supernatants of PBMCs (2.5x10^6/mL) stimulated with HI *A. fumigatus* conidia in the presence or absence of 10 μg/mL IL-1Ra (C) (n=7 for IL-17 n=12 for IL-22), IL-23 (D) (n=12), or anti-IL-23p19 (E) (n=5 for IL-17 n=6 for IL-22). Differences of the means were analysed for significance using the Wilcoxon Signed Rank test.

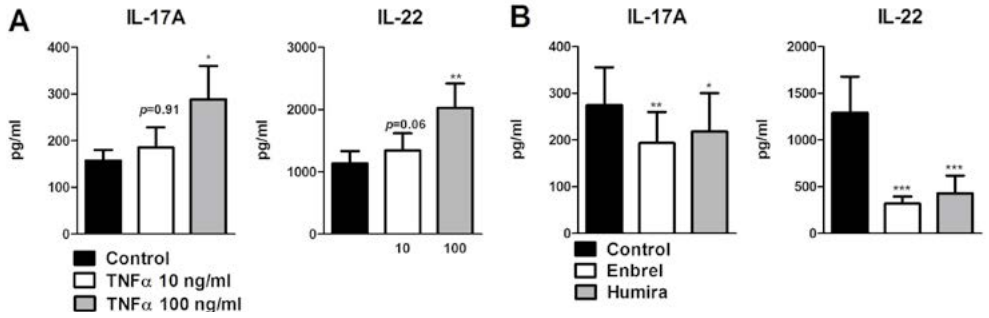

Figure 6 | Role of TNFα in *A. fumigatus*-induced IL-17A and IL-22
(A) IL-17A and IL-22 were measured in the culture supernatants of PBMCs ($2.5x10^6$/mL) (n=10 donors) stimulated for 7 days with 10^7/mL HI *A. fumigatus* conidia with or without recombinant human TNFα. (B) TNFα was blocked by pre-incubation of PBMCs (n=11) for 1 hour with sTNFRII (Enbrel) or human anti-TNFα (Humira). Subsequently the capacity to induce IL-17A and IL-22 was investigated by 7 days stimulation with HI *A. fumigatus* conidia. Differences of the means were analysed for significance using the Wilcoxon Signed Rank test.

Discussion

In the present study we demonstrate that $CD4^+$ T-cells are the main producers of IL-17, IL-22 and IFNγ in human PBMCs upon *Aspergillus* stimulation. Interestingly, CD4 T-helper cells that are capable of producing IL-22 after *Aspergillus* stimulation are not a distinct characteristic subset, but can have a Th1, Th17, or Th22 signature. Similar to IL-17, the production of IL-22 induced by *Aspergillus* is dependent on TLR pathways. While the TLR4 pathway contributing to the production of these cytokines, the TLR2 has an inhibitory effect on *Aspergillus*-induced IL-17 and IL-22 production. In contrast, the TLR2 and TLR4 pathway do not modulate IFNγ production by human PBMCs that are stimulated with conidia. Rather unanticipated, the blockade of the dectin-1/Syk pathway or absence of the dectin-1 receptor did not significantly affect IL-17, IL-22 or IFNγ production induced by *Aspergillus*. In the current study, CR3 is the only receptor that is important for induction of all three cytokines IL-17, IL-22 and IFNγ, and therefore the CR3 pathway could specifically play an important role in the host defence against *Aspergillus*. Furthermore, we provided evidence that IL-17 and IL-22 responses induced by *Aspergillus* are dependent on the IL-1 and TNF pathway.

Using flowcytometric analysis of PBMCs and depletion of CD4 or CD56 cell subsets, we determined that the cytokines IL-17, IL-22 and IFNγ are primarily produced by $CD4^+$ T-cells. Within the $CD4^+$ T-cell populations diverse intracellular cytokine expression was detected. We detected IL-17 single positive and IL-17/IL-22 double positive CD4 cells that match the classically described Th17 cells [13] and IFNγ positive CD4 cells that match the classical Th1 type. Half of the $IFN\gamma^+$ cells were $CD4^+$ cells, whereas the rest of the $IFN\gamma^+$ cells were negative for this T-helper-cell marker. We were able to demonstrate that a significant number of natural killer (NK) cells express IFNγ, which is in line with a previous report that demonstrated that NK cells play an important role in the antifungal response to *A. fumigatus* by releasing IFNγ [43]. However, depletion of $CD56^+$ cells did not alter IFNγ responses, suggesting that NK cells are not important for the production of IL-22 upon stimulation with *Aspergillus*. Furthermore, the $IFN\gamma^+$ $CD56^+$ cells did not expand upon stimulation with

Aspergillus. These data suggest that, in the setting of PBMCs, NK cells do not contribute to the IFNγ response to *Aspergillus*. We found that after stimulation with *Aspergillus* IL-17/IFNγ double positive $CD4^+$ cells were present in low numbers. Therefore, our results demonstrate that the polarization of T-helper responses induced by *Aspergillus in vitro* is different from that induced by the commensal fungus *Candida albicans* [44]. It was demonstrated that *C. albicans* induces a T-cell polarization with high numbers of IL-17/IFNγ double positive T-helper cells. Here we demonstrate that *Aspergillus* stimulation leads to low numbers of this T-cell subset. This is of interest, since these double positive cells have been linked to autoimmunity [44].

We also observed IL-22 single positive cells that might fit the description of Th22 cells [15], as these cells co-expressed TNFα. In addition, large numbers of IFNγ/IL-22 double positive CD4 cells and even IL-17/IL-22/IFNγ triple positive CD4 cells are present. This is to our knowledge the first study that demonstrates that *Aspergillus* can induce single IL-22^+ and IFNγ/IL-22 double positive cells. It remains to be elucidated whether these specific subsets contribute to pathology or protection. A study in mice revealed an important role for IL-22 in the early defence against *Aspergillus* [11], while another study demonstrates contribution to pathology in an allergic aspergillosis model [12]. In the current study, we observed that the pro-inflammatory adaptive cytokine response to *A. fumigatus* is dominated by IL-22, and the primary cellular sources were $CD4^+$ cells. In contrast to the study performed by Gessner and co-workers, our model revealed that IL-22 production by *Aspergillus*-stimulated PBMCs was independent of IL-23. We found that the innate cytokines IL-1 and TNFα played a crucial role in the induction of IL-22, which is in agreement with earlier studies [45]. Previously, it was demonstrated that the IL-22 response also dominated over the IL-17 response in patients with pulmonary tuberculosis [46]. Moreover, IL-22 was shown to be important in the defence against multiple pulmonary pathogens including *Mycobacterium tuberculosis* [14,46,47], *Klebsiella pneumonia* [48], and influenza A virus [16]. Since the host defence against all these pulmonary pathogens strongly relies on IL-22, we hypothesize that IL-22 plays a pivotal role in the human anti-*Aspergillus* pulmonary host defence.

Although TLR2 and TLR4 play an important role in innate immune responses against *A. fumigatus* [28-32], the role of these receptors in the induction of the adaptive immune responses such as the IL-22 response against *Aspergillus* remains to be established. Here we demonstrate that TLR4 plays a role in the induction of IL-17 and IL-22 in response to *Aspergillus*. In contrast, blockade of TLR2 resulted in higher IL-17 and IL-22 production. Therefore, this might indicate that TLR2 plays a role in an inhibitory pathway for the induction of IL-17 and IL-22. These findings are in line with several studies that report an anti-inflammatory role for TLR2 through Th2 skewing [49,50], the induction of anti-inflammatory cytokines by TLR2, and the TLR2-dependent induction of regulatory T-cells [51]. Interestingly, TLR2 is a negative regulator of the Th17 response in a murine pulmonary infection model with *Paracoccidioides brasiliensis* [52], supporting a general role of TLR2 as a negative regulator of Th17 polarization of fungal infections.

The fact that the β-glucan receptor dectin-1 and its downstream kinase Syk play a redundant role in the induction of IL-17, IL-22, and IFNγ by hyphae, which abundantly express β-1,3-glucan, is

rather unanticipated, since the dectin-1 pathway plays an important role in the host defence against invasive aspergillosis [35,53]. In line with our results, it has been demonstrated that IL-17 production by PBMCs with the dectin-1 Tyr238X polymorphism is not different from PBMCs that do not have this SNP [35]. Notably, this SNP influences innate pro-inflammatory cytokines produced by PBMCs in response to *A. fumigatus* [35,53]. In mice, dectin-1 deficiency results in an increased susceptibility to *A. fumigatus* [3], that has been linked to disability to induce protective cytokines like IL-17 [3] and IL-22 [11]. Although it is evident that dectin-1 plays a role in the induction of IL-17 and IL-22 in mice and that it plays a role in the innate host defence against invasive aspergillosis in humans, our studies suggest that IL-17, IL-22 and IFNγ production by $CD4^+$ T-cells in the human host response to *Aspergillus* conidia is not predominantly mediated by dectin-1.

CR3 is a β_2-integrin (CD11b/CD18) that is expressed by monocytes and neutrophils [54,55]. This receptor can recognize self-molecules such as complement, but it can also recognize PAMPs from pathogens such as LPS from *E. coli* [56]. CR3 plays a role in phagocytosis and induction of cytokine responses [57]. Here we show that CR3 is involved in modulating T-helper cytokine responses induced by *A. fumigatus*. To date, the modulation of Th17 and Th1 responses induced by fungi has not been linked to CR3 and future studies are required to further characterize its role in fungal infection. Interestingly, CR3 has been shown to bind β-glucan, which can have modulatory effects on the immune response, suggesting that fungal components can modulate pro-inflammatory T-helper responses (Th1 and Th17) through CR3 [58]. Immunofluorescent staining of β-glucan revealed a very low expression on *Aspergillus* conidia, therefore β-glucan seems to be unlikely the main CR3 ligand in the recognition of *Aspergillus*.

In the present study we compared the IL-1β and IL-23 cytokine profiles produced by PBMCs stimulated with *A. fumigatus* and related this to the induction of IL-17 and IL-22. *Aspergillus* induced relatively low IL-17 levels, which was rather surprising since *A. fumigatus* induced a significant number of IL-17 positive CD4 cells. These observations are in line with a previous report, which demonstrates that *A. fumigatus* is a poor inducer of IL-17 and is even capable of inhibiting the IL-17 response by interfering with the tryptophan metabolism [7]. One possible explanation for the low IL-17 production could be that *Aspergillus* does not induce a significant IL-23 response, since IL-23 production was undetectable in *A. fumigatus* stimulations. Interestingly, supplementation of IL-23 to the *Aspergillus* stimulated PBMCs boosted the IL-17 response, which is in line with an earlier report which showed that IL-23 can augment IL-17 responses in *Aspergillus*-infected mice [3]. Although, *Aspergillus* did not induce IL-23 in PBMCs, it was found earlier that DCs induce high levels of IL-23 upon stimulation with *Aspergillus* and that these DCs can polarize towards both Th1 and Th17 responses [37,59]. However, when we used monocyte-derived DCs instead of PBMCs in our experimental setup we were not able to detect any IL-23 or IL-1β in response to *Aspergillus* (data not shown). We further demonstrate that *Aspergillus*-induced IL-17 and IL-22 responses were dependent on IL-1. Notably, although IL-1β was present and the Th17 response was almost completely dependent on the IL-1 pathway, *Aspergillus* did not abundantly induce IL-17/$IFN\gamma^+$cells. This is in contrast with a previous study that suggests that IL-1β is responsible for the development of $IL\text{-}17^+IFN\gamma^+$ T-helper cells in response to the fungal

pathogen *C. albicans* [44]. These data provide evidence that next to IL-1, another pathway, such as the IL-23 pathway, must be triggered in order to induce IL-17^{+}IFNγ^{+} cells.

Another striking observation is the role of TNF in modulating pro-inflammatory Th17 responses induced by *A. fumigatus*. Blocking TNF lowered the IL-17 production in response to *A. fumigatus*, moreover the effects were most prominent on IL-22 production. These observations are in line with previous reports, which demonstrated that IL-22 production can be triggered by TNFα [15,60,61], and is in line with the observation that anti-TNFα treatment can reduce IL-17 levels in patients [42]. So far, the role of TNFα in pathogen-induced IL-22 production has not been described. This observation could be highly relevant given the widespread use of anti-TNF therapy. Notably, the use of anti-TNF [62-64] and polymorphisms in *TNFR1* or decreased *TNFR1* mRNA expression [64,65] have been linked to increased susceptibility to invasive aspergillosis, underlining the importance to identify the impact of TNF blockade on anti-*Aspergillus* host responses, such as lowering IL-22 production in response to *A. fumigatus* as described in the present study.

In conclusion, we demonstrate that the T-helper cytokines IL-17, IL-22, and IFNγ in response to *A. fumigatus* are primarily produced by CD4 T-cells, and that these cytokines are not limited to a specific subset but can be produced by a variety of polarized T-helper subsets. TLR4 and CR3 and the cytokines IL-1, IL-23, and TNFα are of specific importance for *Aspergillus*-induced IL-17 and IL-22, whereas we observed that the dectin-1/Syk pathway is redundant for this response. Collectively, these findings contribute to a better understanding of the human adaptive host defence against *A. fumigatus,* and provide new knowledge that contributes to the development of targeted adjunctive immunotherapeutic regiments in patients with invasive aspergillosis.

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Supplementary material

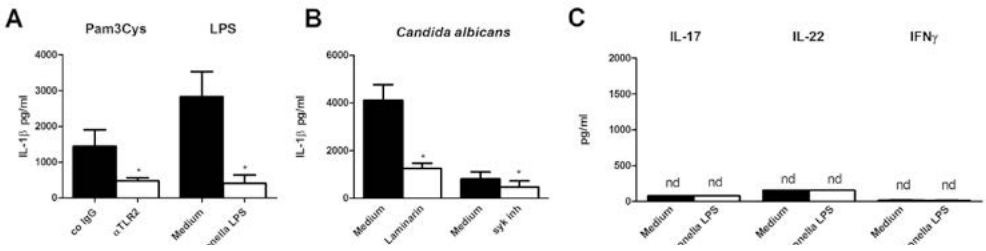
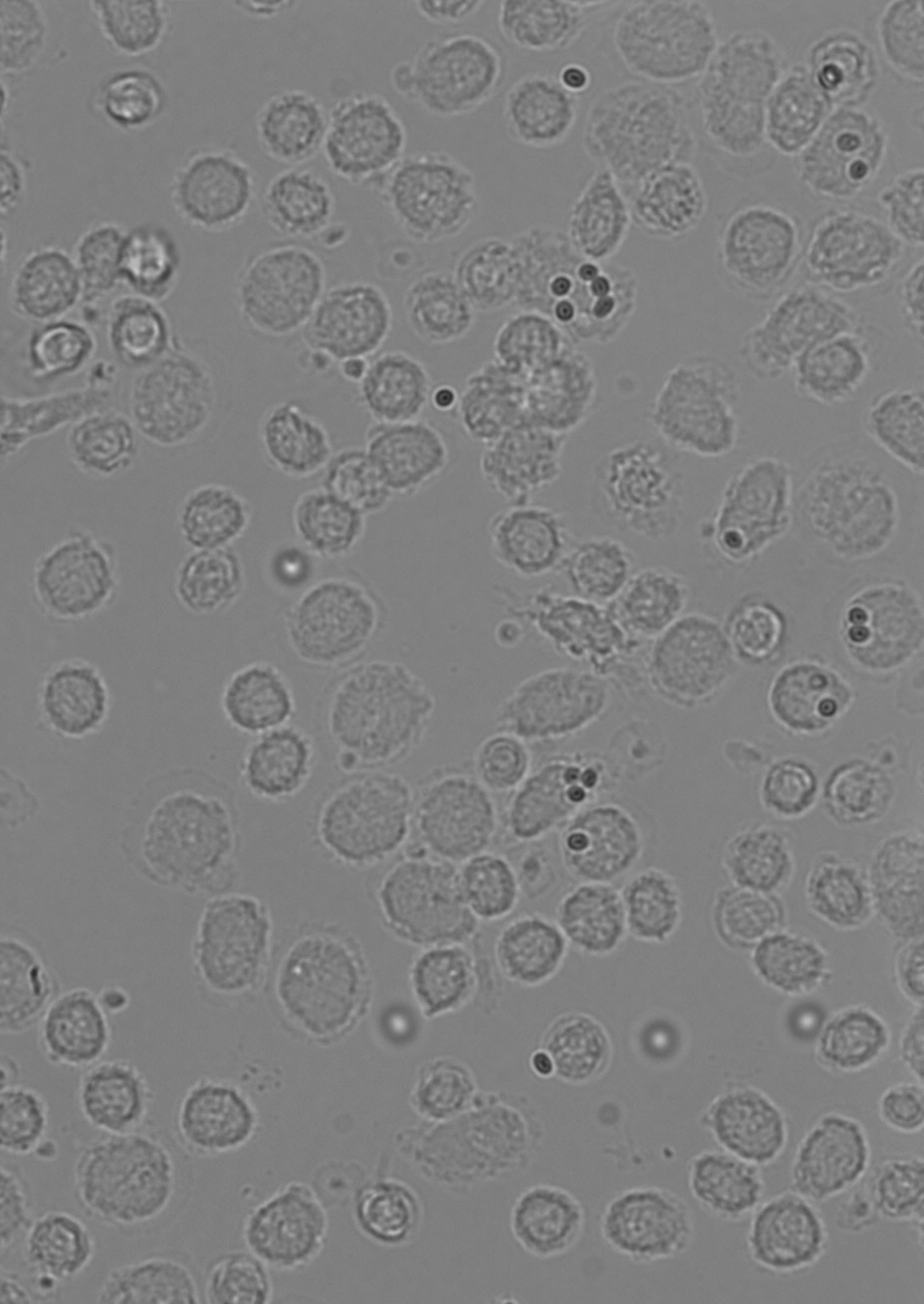


Figure S1 |
(A,B) IL-1β levels in culture supernatants of PBMCs ($2.5x10^6$/mL) (n=6) stimulated with 1μg/mL Pam3Cys, 10ng/mL LPS or $1x10^6$/mL heat-killed *Candida albicans* in the presence or absence of TLR2 neutralizing antibodies (10 μg/mL), isotype control, *Bartonella quintana* LPS (20ng/mL), Laminarin (50 ng/mL) or Syk inhibitor (50mM) respectively (C) IL-17, IL-22 and IFNγ levels in culture supernatants of PBMCs ($2.5x10^6$/mL)(n=8 donors) stimulated with 20 ng/mL LPS from *Bartonella quintana*. Data were analysed using the Wilcoxon signed rank test and are presented as mean ± SEM (*=p<0.05).



Chapter 5

Pattern recognition pathways leading to a Th2 cytokine bias in ABPA patients

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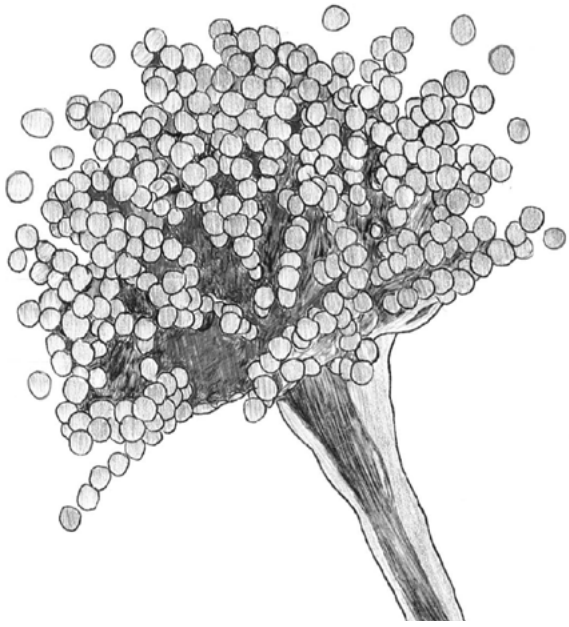

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Abstract

Background | Allergic bronchopulmonary aspergillosis (ABPA) is characterized by an exaggerated T-helper (Th)2 response to *Aspergillus fumigatus*, but the immunological pathways responsible for this effect are unknown.

Objective | The aim of this study was to decipher the pattern recognition receptors (PRRs) and cytokines involved in the *Aspergillus*-specific Th2 response, and to study *Aspergillus*-induced responses in healthy controls and ABPA patients.

Experimental procedures | Peripheral blood mononuclear cells (PBMCs) were stimulated with heat-killed *Aspergillus* conidia, various other pathogens, or PRR ligands. PRRs and cytokine pathways were blocked with PRR blocking reagents, sTNFRII (Etanercept) or anti-TNFα (Adalimumab), IL-1Ra (anakinra), or recombinant human IFNγ (Immukine). ELISA and flowcytometry were used to analyze cytokine responses.

Results | *Aspergillus* was the only pathogen that stimulated the Th2 cytokines IL-5 and IL-13, while Gram-negative bacteria, Gram-positive bacteria, *Candida albicans,* chitin, β-glucan, or Toll-like receptors (TLR) ligands did not. Depletion of $CD4^+$ cells abolished IL-13 production. Blocking complement receptor 3 (CR3) significantly reduced IL-5 and IL-13, while blocking TLR2, TLR4 or dectin-1 had no effect. ABPA patients displayed increased *Aspergillus*-induced IL-5 and IL-13, and decreased IFNγ production compared to healthy controls. All biological agents tested showed the capability to inhibit Th2 responses, but also decreased *Aspergillus*-induced IFNγ.

Conclusions | *Aspergillus* conidia are unique in triggering Th2 responses in human PBMCs, through a CR3-dependent pathway. ABPA patients display a significantly increased *Aspergillus*-induced Th2/Th1-ratio that can be modulated by biologicals. These data provide a rationale to explore IFNγ therapy in ABPA as a corticosteroid-sparing treatment option, by dampening Th2 responses and supplementing the IFNγ-deficiency at the same time.

Introduction

Allergic bronchopulmonary aspergillosis (ABPA) is a hypersensitivity reaction against the ubiquitous fungus *Aspergillus fumigatus* [1]. Innate immune cells, such as lung epithelia cells or alveolar macrophages initiate this inflammatory reaction [2]. These innate immune cells sense fungi through pattern recognition receptors (PRRs), like Toll-like receptors (TLRs), C-type-lectin receptors (CLRs) or NACHT-LRR-receptors (NLRs) [3]. Activation of these receptors by *Aspergillus* leads to induction of cytokines, recruitment of other immune cells, and can eventually trigger adaptive immune responses such as T-helper (Th) responses. A robust induction of the Th1 response during *Aspergillus* infection is associated with protection and successful clearance [4-6], while elevated Th2 responses impairs fungal clearance [7, 8]. Exaggerated Th2 responses are being held responsible for the detrimental inflammatory reaction observed in ABPA, namely eosinophilia, increased mucus production and immunoglobulin class switching to IgE. This inflammatory reaction subsequently results in airway hyper-responsiveness with typical asthmatic symptoms like bronchial obstruction, coughing and wheezing [9, 10]. The current treatment for ABPA is the administration of oral corticosteroids during exacerbations and control of the underlying asthma with β_2-agonists. Furthermore, antifungal treatment with azoles in ABPA patients could spare the use of corticosteroids [1, 11]. However, due to the potential severe side effects of azoles and the long-term consequences of corticosteroid treatment, new approaches for ABPA therapy are needed.

While most *Aspergillus*-related PRRs have been studied in the context of invasive aspergillosis, limited data exists of the *Aspergillus*-associated molecular patterns and their PRRs responsible for triggering the Th2 response in ABPA. In murine studies, TLR2 and TLR9 have been described to skew the *Aspergillus* induced Th response towards a Th2 profile [12, 13], and chitin exposure has been associated with pulmonary eosinophilia[14]. The current study aims to identify the immunological pathways through which *Aspergillus* induces a Th2 response in humans. Furthermore, the cytokine profile of a cohort of ABPA patients was investigated for assessing the Th1/Th2 balance. The capacity of treatment with biological therapy to modulate *Aspergillus*-induced T-helper responses was tested *in vitro,* with the goal to find innovative corticosteroid-sparing treatment options for ABPA patients.

Experimental procedures

Volunteers and patients | Blood was collected from healthy volunteers or patients by venous blood puncture after informed consent. Three dectin-1 deficient patients with a homozygous Y238X mutation in exon 6 of *CLEC7A* gene (the gene encoding dectin-1) donated blood, two of them twice at two different time points. In these patients diminished expression of dectin-1 and failure to induce a cytokine response to β-glucan was demonstrated previously [15]. Seven asthma patients and nine patients diagnosed with ABPA according to the ABPA diagnostic criteria as described elsewhere [3] were asked for blood donations, two of whom had cystic fibrosis with a homozygous mutation in the *CFTR* gene (Table 1).

PRR ligands, blockers and other stimuli | *Escherichia coli* LPS (10 ng/mL) (TLR4 ligand, *E. coli* serotype O55:B5, Sigma-Aldrich St. Louis, MO USA), Pam3Cys (10 µg/mL) (TLR2 ligand, EMC microcollections, Tübingen, Germany), Poly I:C (10 µg/mL) (TLR3 ligand, Cayla-Invivogen, Toulouse, France), flagellin (10 µg/mL) (TLR5 ligand, Cayla-Invivogen, Toulouse, France), N-acetylmuramyl-ananyl-D-isoglutamine (MDP) (10 µg/mL) (NOD2 ligand, Sigma-Aldrich), phytohemagglutinin (PHA) (10 µg/mL) (Sigma-Aldrich). *Escherichia coli* ATCC 35218 (*E. coli*) (1x10^7/mL); *Staphylococcus aureus* clinical isolate (*S. aureus*) (1x10^7/mL); *Mycobacterium tuberculosis* sonicate H37Rv bub DL 6122005 (*M. tuberculosis*) (10 µg/mL); *Borrelia burgdorferi* ATCC 35210 (*B. burgdorferi*) (1x10^6/mL); *Candida albicans* ATCC MYA-3573 (UC820) (*C. albicans*) (1x10^6/mL); *Aspergillus fumigatus* clinical isolate V05-27 (*A. fumigatus*), resting conidia and hyphae (1x10^7/mL,) were cultured and isolated as described in a previous study [16].

Fungal cell wall components: β-1,3-D-glucan (β-glucan) (10 µg/mL) was kindly provided by Prof. David Williams (Tennessee University), and α-1,3-glucan, α-1,3-1,4-glucan (either dissolved in PBS or DMSO), chitin (*Aspergillus niger*) (all 10 µg/mL) and galactosaminogalactan (GAG) (7,5 µg/mL) were a gift from Prof. Jean-Paul Latgé (Pasteur Institute, Paris).

Bartonella quintana LPS was prepared and purified as described elsewhere [18] and used as a TLR4 inhibitor (20 ng/mL) [19]; isotype control mouse IgG1 (10 µg/mL) (eBioscience, Halle-Zoersel, Belgium); anti-TLR2 (10 µg/mL) (eBioscience); laminarin (50 ng/mL) [17]; a spleen tyrosine kinase (Syk) inhibitor (50 nM, Merck, Darmstadt, Germany); isotype control goat IgG (10 µg/mL) (R&D Systems Minneapolis, MN, USA); anti-human β_2-integrin (anti-CR3) (10 µg/mL) (R&D Systems); anti-human OX40ligand (anti-OX40L) (10 µg/mL) (R&D Systems); cytochalasine D (1 µg/mL, dissolved in DMSO) (Enzo life sciences, Antwerpen, Belgium); IL-1 receptor antagonist (IL-1Ra) (10 µg/mL) (Amgen, Inc., Thousand Oaks, CA, USA); soluble TNFRII (Etanercept) (100 µg/mL), human anti-human TNFα (Adalimumab), (100 µg/mL), both kindly provided by Dr. Marije Koenders (Department of Rheumatology Radboud University Nijmegen Medical Centre the Netherlands); Interferon-gamma 1b (Immukine) (50 ng/mL) (Boehringer-Ingelheim, Alkmaar, The Netherlands).

PBMCs isolation | Venous blood was drawn in 10 mL EDTA tubes. The blood was diluted 1:1 with Phosphate Buffered Saline (PBS). Subsequently PBMCs were isolated using Ficoll-paque (GE healthcare, Zeist, The Netherlands) density gradient centrifugation. The PBMCs layer was collected and washed twice in cold PBS. Cells were reconstituted in RPMI-1640 culture medium (Dutch modification, Gibco, Invitrogen, Breda, The Netherlands) supplemented with 10 µg/mL gentamicin, 10 mM L-glutamine and 10 mM pyruvate (Gibco). The cells were counted with a particle counter (Beckmann Coulter, Woerden, The Netherlands) and the concentration was adjusted to 1x10^7 cells/mL.

Cell depletion | PBMCs were incubated with magnetic anti-CD4, anti-CD56 and anti-CD19 beads and purified using MACS depletion columns (LD, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the instructions supplied by the manufacturer to generate $CD4^-$ (T-cells depleted), $CD56^-$ (NK-cells depleted) or $CD19^-$ (B-cells depleted) PBMC fractions. Next to the labelled cells, also an unlabelled PBMC fraction was run over the depletion column and used as a mock treatment control.

PBMCs stimulation | PBMCs were plated in a 96-well round-bottom plate (Corning, NY, USA) at a final concentration of $2.5x10^6$/mL in an end volume of 200 µL per well. All stimulations were performed in the presence of 10% human serum. Serum was either complement active if not otherwise indicated or heat-inactivated by incubation for 30 minutes at 56°C in a water bath according to a commonly used protocol [18]. Mannose-binding lectin (MBL) deficient serum was obtained from a patient with MBL level of 0.09 µg/mL. After 1 hour pre-incubation with inhibitor or medium, stimuli or medium were added. Cells were incubated at 37°C with 5% CO_2, after 7 days supernatants were collected and stored at -20°C.

Cytokine measurements | IL-5, IL-13, IFNγ and IL-10 were measured in the cell culture supernatants using a commercial ELISA kit (IL-5 and IL-13: R&D Systems; IFNγ and IL-10: Sanquin) according to the instructions supplied by the manufacturer.

Intracellular staining and flowcytometric analysis | After 7 days incubation, cells were re-stimulated for 4-6 hours with 200 µL of RPMI supplemented with gentamicin, L-glutamine and pyruvate and PMA (50 ng/mL) (Sigma-Aldrich), ionomycin (1 µg/mL) (Sigma-Aldrich), Golgiplug (BD Biosciences, Breda, the Netherlands) and 10% human serum. Cells were stained extracellular using PECy7–conjugated anti-CD4 (BD Biosciences), PECy7–conjugated anti-CD8 (BioLegend, San Diego), or PECy7–conjugated anti-CD56 (Beckman Coulter) and ECD-conjugated monoclonal anti-CD3 antibody (Beckman Coulter, Krefeld, Germany). Fixation and permeabilization was performed with Cytofix/Cytoperm solution (eBioscience) according to the instructions supplied by the manufacturer. Cells were stained intracellular with anti-IL-4 (FITC-conjugated), anti-IL-5 (PE-conjugated) and anti-IL-13 (APC-conjugated) (BD Bioscience). Fixated cells were measured with a FC500 flowcytometer (Beckman Coulter) and the data were analysed using CXP analysis software v2.2 (Beckman Coulter).

OX40L expression | RNA was isolated from $1x10^6$ PBMCs after stimulation for 48 hours with *Aspergillus* conidia either in the presence of medium or Adalimumab or IL-1Ra using Trizol Reagent (Invitrogen) according to a protocol supplied by the manufacturer. RNA (500 ng) was reverse transcribed into cDNA using the iScript cDNA synthesis kit (Hercules, Bio-Rad Laboratories, CA). Quantitative PCR (qPCR) analysis was performed using SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA) and the Applied Biosystems 7300 real-time PCR system. As PCR protocol the following conditions were used: 2 min 50°C, 10 min 95°C followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. For the amplification of hOX40L the primers 5'-TCCCCTCTCTTAGGTGCTCA-3' and 5'-GGCTGGTGCATAGCAGAAAT-3' were used. To correct for differences in loading concentrations of RNA between the different conditions, qPCR results were corrected with the housekeeping gene β2 microglobulin (β2m) amplified using the primers 5'- ATGAGTATGCCTGCCGTGTG-3' and 5'-CCAAATGCGGCATCTTCAAAC-3'. Primer efficacy was evaluated using a standard curve. OX40L Ct values were compared with the β2m Ct using the formula $2^{-\Delta Ct}$ and the foldchange was calculated by setting the *Aspergillus*-stimulated samples as 1 to determine the effect of anti-TNFα (Adalimumab) and IL-1Ra on OX40L expression.

Genotyping | Genomic DNA from healthy Caucasian controls was isolated using the Gentra Pure Gene blood kit (Qiagen, Hilden, Germany) according to the instructions supplied by the manufacturer. The specific SNP identifiers (rs numbers) were extracted from a recent publication describing SNPs associated with ABPA [19]. Genotypes of the different SNPs were obtained from the Illumina immunochip which has previously been run according to the manufactures protocol [20]. Beforehand, a quality filter was applied which automatically excluded SNPs with either a low call rate (<99%), SNPs that were not in Hardy-Weinberg equilibrium (p<0.01) or that had a reported minor allele frequency of less than 0.01.

Statistical analysis | The Mann-Whitney-U test was used to detect differences between healthy controls and patients or between different genotypes. The Wilcoxon signed rank test was used to determine differences between stimulation with and without inhibitors of PRRs, cytokines or cytokine inhibitors. A p-value of <0.05 was considered Statistically significant (*=p<0.05, **=p<0.01 and ***=p<0.001). Graphs represent cumulative results of all performed experiments and are presented as mean ± standard error of the mean (SEM). Data were analysed with GraphPad Prism v 5.0.

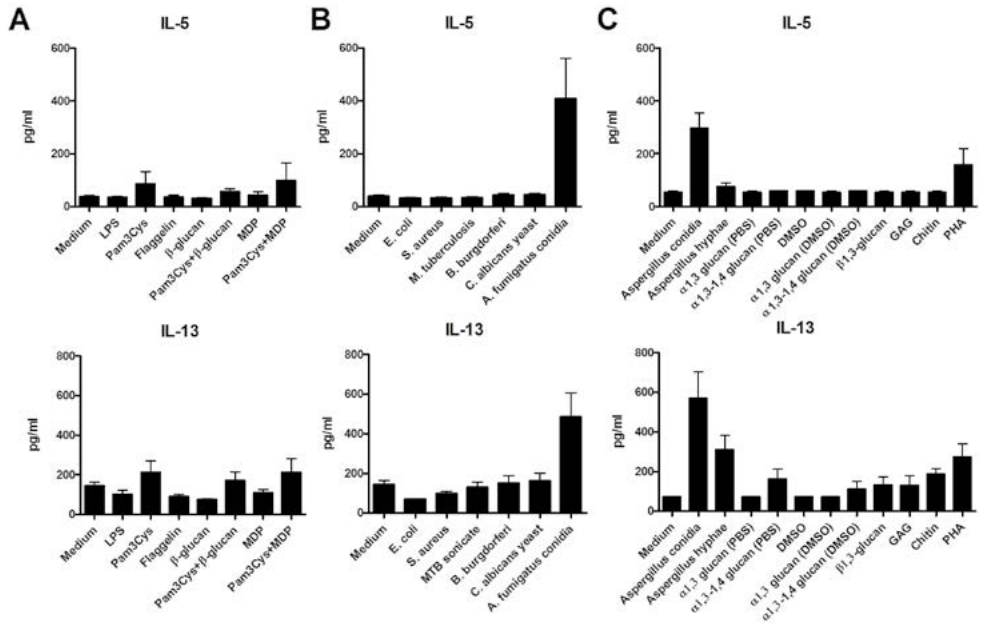

Figure 1 | *Aspergillus* conidia induce a Th2 response, in contrast to other pathogens or PRR ligands
IL-5 and IL-13 concentrations were measured in cell culture supernatants of PBMCs of healthy controls (2.5x10^6/mL, n=6 donors) by ELISA (IL-5 detection level 24 pg/mL, IL-13 detection level 71 pg/mL) after stimulation with (A) TLR4 ligand (LPS), TLR2 ligand (Pam3Cys), TLR3 ligand (Poly I:C), TLR5 ligand (flaggelin), NOD2 ligand (MDP) or dectin-1 ligand (β-glucan) (B) Various pathogens like *E. coli*, *S. aureus*, *M. tuberculosis* sonicate (H37Rv), *B. burgdorferi*, *C. albicans* yeast and *A. fumigatus* conidia (C) *A. fumigatus* conidia and hyphae as well as differenT-cell wall components like α-1,3-glucan, α-1,3-1,4-glucan, β-1,3-glucan, galactosaminogalactan, chitin PHA.

Results

***Aspergillus* conidia induce a Th2 response, in contrast to other pathogens or PRR ligands**

We investigated which PRRs are involved in the initiation of a Th2 response by stimulating PBMCs with different specific ligands for TLR4, TLR2, TLR5, NOD2 or dectin-1, and measuring IL-5 and IL-13 (Figure 1A). None of these ligands or combinations of TLR2 with ligands for dectin-1 or NOD2 was able to induce IL-5 or IL-13. We thereafter screened a panel of pathogens consisting of extra- and intracellular bacteria, as well as two types of fungi for their capacity to induce Th2 cytokines (Figure 1B). The whole bacteria *E. coli, S. aureus* or *B. burgdorferi,* as well as sonicated *M. tuberculosis* were unable to induce IL-5 and IL-13, while these pathogens induce other immune responses such as IL-17 and IFNγ in these conditions (data not shown). Among the fungal pathogens, the Th2 response was restricted to the stimulation with *A. fumigatus* conidia, while *C. albicans* yeast did not induce IL-5 and IL-13. Moreover, *Aspergillus* hyphae induced relatively low IL-5 production (76.4 pg/mL hyphae vs. 296 pg/mL conidia) and IL-13 (310 pg/mL hyphae vs. 568 pg/mL conidia). Stimulation with the fungal cell wall components α-1,3-glucan and α-1,3-1,4-glucan, β-1,3-glucan, galactosaminagalactan (GAG) or chitin did not induce IL-5, while IL-13 production was slightly induced by α-1,3-1,4-glucan or chitin (Figure 1C). PHA was used as a mitogenic stimulus to detect T-cell memory activation, and induced low levels of Th2 cytokines.

***Aspergillus*-induced IL-5 and IL-13 are dose and time dependently produced by $CD4^+$ T-cells**

To study the kinetics and dose dependency of IL-5 and IL-13 induced by *Aspergillus* conidia, we performed a time course of 7 days with 3 different concentrations ranging from $1x10^5$ to $1x10^7$ conidia/mL (Figure 2A). IL-4 was undetectable in all stimulations (data not shown). In contrast, IL-5 and IL-13 were detectable from day 4 onwards and increased steadily. Only the highest dosage of $1x10^7$ conidia/mL induced IL-5 and IL-13, while dosages below this threshold induced cytokine values barely detectable above the detection limit.

To investigate which cells are responsible for the production of IL-4, IL-5 and IL-13 induced by *Aspergillus*, flowcytometric analyses with intracellular staining for IL-4, IL-5 and IL-13 were performed on PBMCs that were cultured for 7 days in the presence of *Aspergillus* conidia. Within the Th2 cytokine positive population, $CD4^+$ cells were the main representatives (72.9% of IL-4^+, 61.7% of IL-5^+ and 68.5% of IL-13^+ cells) (Figure 2B). Surface staining for CD8, CD56 and CD19 revealed the $CD8^+$ cells as the second biggest subpopulation contributing to the IL-5 and IL-13 production (Figure 2C). To determine which cell populations within PBMCs contribute to the IL-5 and IL-13 secretion induced by *Aspergillus* stimulation, PBMCs were depleted of $CD4^+$, $CD56^+$ or $CD19^+$ cells (Figure 2D). Mock-treated PBMCs lost their capacity to produce IL-5, which made it impossible to evaluate the effect of depleting a special subpopulation on IL-5 production (Figure S1). Depletion of the $CD4^+$ cells led to a reduction of *Aspergillus*-induced IL-13 production to undetectable levels, while depleting $CD56^+$ or $CD19^+$ cells did not affect IL-13 production. These data suggest that $CD4^+$ T-lymphocytes are the main producers of Th2 cytokines induced by *Aspergillus* conidia.

***Aspergillus*-induced IL-5 and IL-13 response is dependent on CR3 and phagocytosis**

Since most PRRs have been studied in the context of invasive aspergillosis [3,21] only little is known about the PRRs and pathways involved in the pathogenesis of ABPA. We investigated the role of TLR2, TLR4, Dectin-1 and CR3 pathways in *Aspergillus*-induced Th2 responses in human PBMCs. PBMCs were stimulated with *Aspergillus* conidia in the absence or presence of specific inhibitors for the PRR pathways. Inhibition of TLR4 did not alter IL-5 production, while IL-13 was slightly reduced (mean 15.7% reduction, Figure 3A). Although TLR2 has been described to polarize *Aspergillus*-specific T-cell responses towards Th2 [22,23], neither IL-5 nor IL-13 was affected when TLR2 was blocked (Figure 3B). Dectin-1 is known to recognize the fungal cell wall component β-glucan [24] and is known to promote immunopathology in the lung during fungal allergy [25]. However, blocking dectin-1 with laminarin (Figure 3C), or inhibition of Syk, the downstream signalling kinase of the dectin-1 receptor, did not alter the Th2 response induced by *Aspergillus* (Figure 3D). Additionally, PBMCs isolated from three dectin-1 deficient patients produced IL-5 and IL-13 levels comparable to healthy controls (Figure 3E). Subsequently, we investigated CR3 (CD11b/CD18), a β_2-integrin expressed on neutrophils and monocytes, since it has been described that CR3 is also able to recognize β-glucan [26]. Blockade of CR3 inhibited the *Aspergillus*-induced Th2 response completely (Figure 3F). We did not observe different cytokine production when the serum was de-complemented by heat-inactivation (Figure 3G).

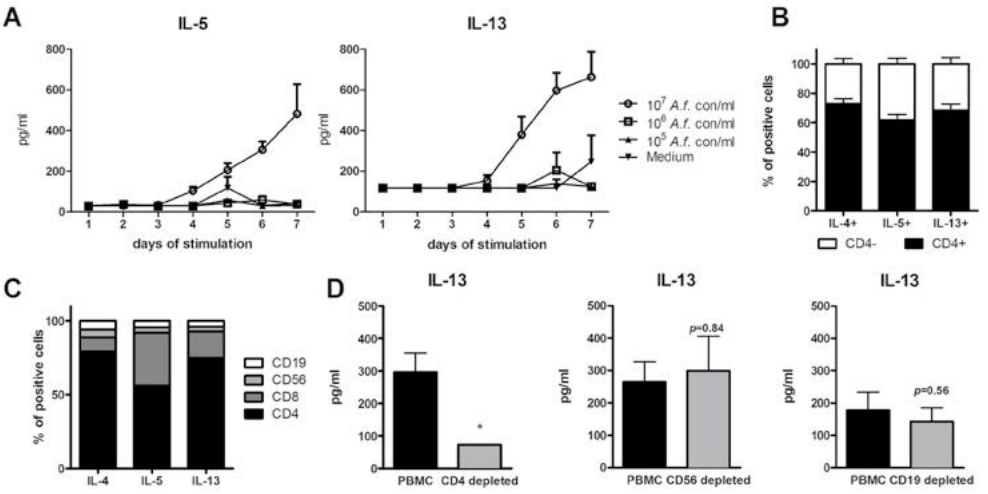

Figure 2 | *Aspergillus*-induced Th2 cytokines are dose and time dependently produced by CD4⁺ cells

(A) PBMCs (2.5x10^6/mL, n=4 donors) were stimulated with increasing concentrations of *Aspergillus* conidia (1x10^5/mL, 1x10^6/mL, 1x10^7/mL) for 1-7 days and IL-5 and IL-13 was measured in the cell culture supernatant by ELISA. (B-C) Cells were stained extracellular for CD4, CD8, CD56 and CD19 and intracellular for IL-4, IL-5 and IL-13. (B) CD4$^+$ and CD4$^-$ cells (n=10) within the IL-4, IL-5 or IL-13 positive cells. (C) CD4$^+$-, CD8$^+$-, CD56$^+$ and CD19$^+$-cells within the *Aspergillus*-specific IL-4$^+$-, IL-5$^+$- and IL-13$^+$-populations (normalized to 100%, one representative donor) (D) PMBCs were depleted either for CD4$^+$, CD56$^+$ or CD19$^+$ cells (white bars) or run over the depletion columns unlabelled (black bars) and stimulated with *Aspergillus* conidia. IL-13 was measured in the cell culture supernatant by ELISA (n=6 donors). Statistical analysis was performed with the Wilcoxon Signed Rank test.

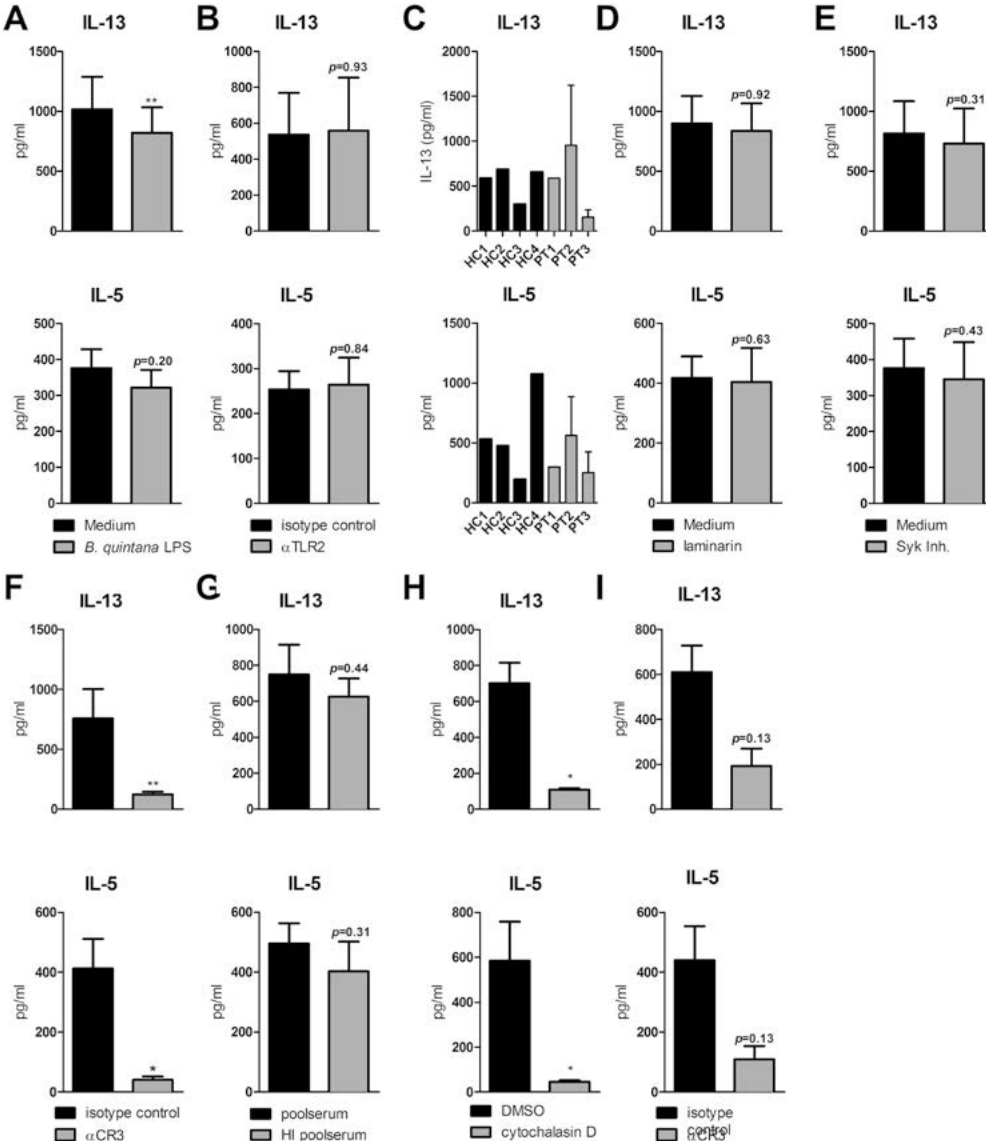

Figure 3 | *Aspergillus*-induced IL-5 and IL-13 response is dependent on CR3 and phagocytosis
PBMCs were pre-incubated for 1h with different blockers and stimulated with *Aspergillus* conidia. IL-5 and IL-13 were measured in the cell culture supernatant (black bars: *Aspergillus* conidia stimulation with medium or control, grey bar: *Aspergillus* conidia stimulation after blocking). (A) TLR4 blocking with *B. quintana* LPS (IL-5 n=9 donors, IL-13 n=8 donors). (B) TLR2 blocking with αTLR2 antibody (IL-5 n=6 donors, IL-13 n=7 donors). (C) PBMCs of three dectin-1 deficient patients compared with four healthy controls. (D) Dectin-1 blocking with laminarin (n=10 donors). (E) Syk inhibition (IL-5 n=9 donors, IL-13 n=7 donors). (F) CR3 blocking with αCR3 antibody (IL-5 n=7 donors, IL-13 n=9 donors). (G) PBMC stimulation with 10% active human serum compared with 10% heat-inactivated human serum (IL-5 n=5 donors, IL-13 n=6 donors). (H) Blocking phagocytosis with cytochalasine D (n=6 donors). (I) CR3 blocking with αCR3 antibody (IL-5 and IL-13 n=4 donors) in the presence of MBL deficient serum. Statistical analysis was performed with the Wilcoxon Signed Rank test.

Since CR3 is involved in phagocytosis [27], we blocked phagocytosis by inhibiting actin polymerisation by cytochalasine D, which significantly reduced IL-5 and IL-13 production (Figure 3H). High plasma levels of MBL are associated with bronchial asthma with allergic rhinitis and ABPA [28], therefore we wanted to investigate whether the recognition of *Aspergillus* conidia with subsequent Th2 induction via CR3 is MBL dependent. *Aspergillus*-specific Th2 cytokines in the presence of MBL deficient serum were significantly decreased after blocking CR3, suggesting that signalling via CR3 leading to Th2 responses is independent of MBL (Figure 3I). These data demonstrate that the IL-13 response induced by *Aspergillus* in human PBMCs is partially dependent on TLR4, while both IL-5 and IL-13 are dependent on CR3 and phagocytosis.

ABPA patients have an *Aspergillus*-specific increased Th2/Th1 ratio

PBMCs isolated from nine ABPA patients were stimulated with *Aspergillus* conidia and IL-5, IL-13 and IFNγ production was measured. Compared to non-allergic healthy controls, *ex vivo Aspergillus*-induced Th2 cytokines was stronger in ABPA patients, which was Statistically significant for IL-5 but not for IL-13 (Figure 4A). In contrast, *Aspergillus*-induced IFNγ production by PBMCs was significantly lower in ABPA patients when compared to healthy controls (Figure 4A). Interestingly, IFNγ was significantly lower after stimulation with both *Aspergillus* conidia and hyphae (data only shown for conidia). When we compared the IL-5/IFNγ and IL-13/IFNγ ratios in ABPA patients and controls, we observed that they were significantly different and discriminated ABPA patients from healthy controls (Figure 4B, C).

Table 1 | Demographic table of ABPA patients

No.	Sex Age (y)	asthma/CF	total IgE *Asp*-IgE (U/mL)	*Asp*-IgG (mg/mL)	Eo	Be	steroids use	azole use
ABPA 1	F 22	dF508del /dF508del	60 1.5	48.60	1.06	yes	yes: ciclosenide, inh 160 mg	yes
ABPA 2	M 21	dF508del /dF508del	229 13.9	40	0.79	yes	no	yes
ABPA 3	M 63	asthma	1406 25.1	353	2.44	yes	yes: prednisone, 5mg/d	no
ABPA 4	F 78	asthma	4515 67.7	n.d.	0.17	n/a	no	yes
ABPA 5	F 77	asthma	1263 46.4	120	0.88	yes	no	no
ABPA 6	M 67	asthma	2710 29	128	0.88	discrete	no	yes
ABPA 7	M 66	asthma	2508 15.1	187	n/a	yes	yes: prednisone, 10mg/d	no
ABPA 8	F 70	asthma	3202 40.7	196	0.02	discrete	yes: prednisone, 15mg/d	no
ABPA 9	M 77	asthma	3343 46.4	51	1.49	yes	yes: prednisone, 10mg/d	no

Age, sex, underlying disease (asthma or cystic fibrosis), bronchiectasis (Be), eosinophil count (Eo), total IgE (U/mL), *Aspergillus*-specific IgE (*Asp*-IgE)(U/mL), *Aspergillus*-specific IgG (*Asp*-IgG)(mg/mL) and concomitant medication (corticosteroids and azoles) of the ABPA patients enrolled in this study are listed.

To determine whether the elevated Th2/Th1 ratio in ABPA is due to the underlying asthma itself or due to *Aspergillus* sensitization, we investigated *Aspergillus*-specific ratios in asthma patients with and without an elevated *Aspergillus*-specific IgE (Table S1). PBMCs isolated from asthma patients with *Aspergillus*-sensitization that were stimulated with *Aspergillus* conidia showed a higher *Aspergillus*-specific Th2 response than the asthma group without *Aspergillus*-sensitization and the control group (Figure 4D). This resulted in an elevated Th2/Th1 ratio comparable to ABPA patients (Figure E). In addition, we investigated whether the anti-inflammatory cytokine IL-10 is involved in the elevated ratios observed in ABPA. Neither in the control, nor in the patient group did we detect IL-10 concentrations (Figure S2).

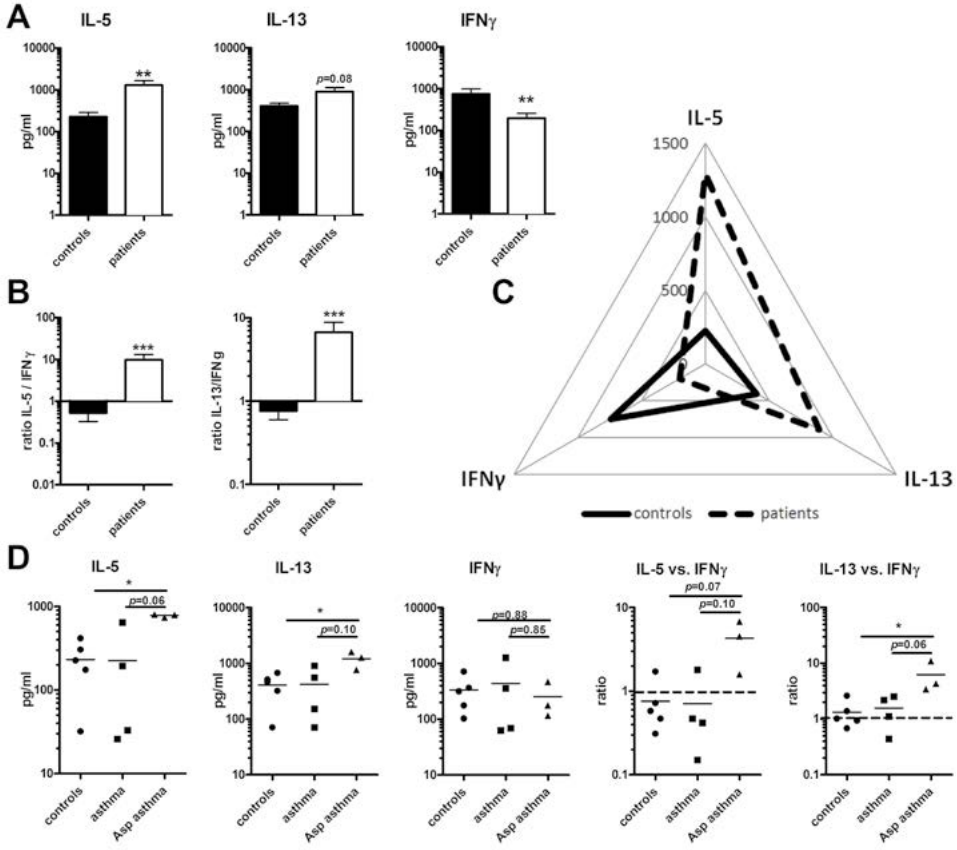

Figure 4 | The Th2/Th1 balance in ABPA patients is shifted towards Th2

(A) PBMCs of 9 ABPA patients were stimulated with *Aspergillus* conidia and compared with PBMCs of healthy controls. IL-5, IL-13 and IFNγ levels were measured in the cell culture supernatant (black bars controls, white bars patients). (B) The Th2/Th1-ratio between IL-5 vs. IFNγ and IL-13 vs. IFNγ was generated by dividing the individual IL-5 or IL-13 level by the individual IFNγ level. (C) The triangle diagram shows the mean of IL-5, IL-13 or IFNγ of every group on the corresponding cytokine axis (continuous line: controls, dotted line: patients). (C) PBMCs isolated from 3 asthma patients with an elevated *Aspergillus*-specific IgE and 4 without elevated *Aspergillus*-specific IgE were stimulated with *Aspergillus* conidia and compared with healthy controls. IL-5, IL-13 and IFNγ levels were measured in the cell culture supernatant. (D) The Th2/Th1-ratio between IL-5 vs. IFNγ and IL-13 vs. IFNγ was generated by dividing the individual IL-5 or IL-13 level by the individual IFNγ level. Statistical analysis was performed with the Mann-Whitney-U test.

Etanercept, Adalimumab, anakinra and IFNγ modulate the *Aspergillus*-induced Th2/Th1 ratio

Since we observed significantly different Th2/Th1 ratios in ABPA patients we wanted to investigate whether available biologicals for treatment of inflammatory disease are capable of modulating the *Aspergillus*-specific Th2/Th1 ratio. We used Etanercept (soluble TNFα receptor II-Fc), Adalimumab (human anti-human TNFα), anakinra (IL-1 receptor antagonist) and Immukine (recombinant human IFNγ) and stimulated PBMCs either with *Aspergillus* conidia in the presence of medium or one of the biologicals. All biologicals significantly suppressed *Aspergillus*-induced IL-5 and IL-13 production in human PBMCs. However, Etanercept, Adalimumab and anakinra also suppressed IFNγ production in response to *Aspergillus* and hence did not correct the IL-5/IFNγ-ratios (Medium: 0.65, Etanercept l: 0.61, Adalimumab: 0.92, anakinra: 1.82) and IL-13/IFNγ-ratios (Medium: 1.49, Etanercept: 1.41, Adalimumab: 1.89, anakinra: 2.77). Therefore, it was only recombinant IFNγ that was able to restore the increased Th1/Th2 ratio by down-regulating IL-5 and IL-13, but not IFNγ.

The co-stimulatory molecule OX40L is known to be involved in inflammatory Th2 responses triggered by thymic stromal lymphopoetin (TSLP)-activated dendritic cells in ABPA patients [29]. We therefore investigated whether OX40L is also involved in the Th2 responses induced by *Aspergillus*

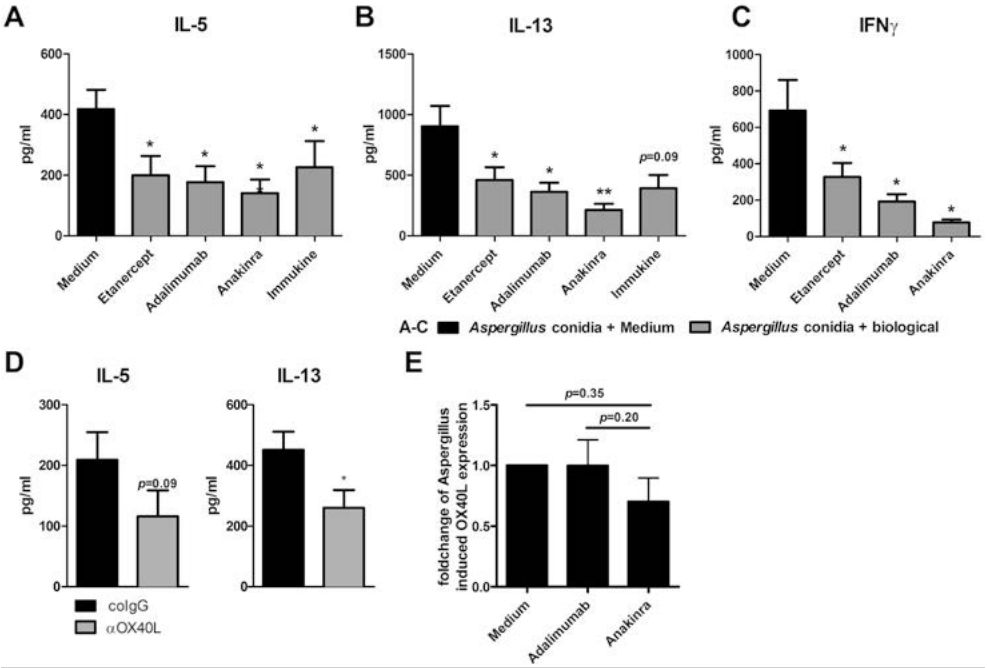

Figure 5 | Etanercept, Adalimumab, Anakinra and IFNγ modulate the *Aspergillus*-induced Th2/Th1 ratio
PBMCs of healthy controls were stimulated with *Aspergillus* conidia either the presence of medium (black bars) or one of the biological drugs (grey bars) like Etanercept (soluble TNFα receptor type II), Adalimumab (human TNFα antibody), anakinra (IL-1Ra) or recombinant IFNγ. (A) IL-5, (B) IL-13 and (C) IFNγ were measured with ELISA in the cell culture supernatant. (D) IL-5 and IL-13 measurement after blocking OX40L with αOX40L antibody (IL-5 and IL-13, n=9 donors). (E) OX40L expression in PBMCs of healthy controls stimulated with *Aspergillus* conidia either in the presence of adalimumab or anakinra (n=6). Statistical analysis was performed with the Wilcoxon Signed Rank test.

in human PBMCs. Blocking OX40L resulted in significantly decreased *Aspergillus*-induced IL-13 production (Figure 5D). Since TNFα and IL-1 can modulate the expression of OX40L [30,31], we analysed whether blocking TNFα or IL-1 would result in a different expression of OX40L in PBMCs. We observed no significant modulation of the *Aspergillus*-induced OX40L expression in the presence of adalimumab or anakinra (Figure 5E).

Polymorphisms influencing Aspergillus-specific Th2/Th1 ratios

Single nucleotide polymorphisms (SNPs) in the *IL10*, *IL4Rα*, *MBL*, and *SFTPA2*, gene have been associated with an increased susceptibility to ABPA [19]. We took a systematic approach to investigate whether reported polymorphisms that are associated with ABPA could influence *Aspergillus*-specific Th2/Th1 ratios (table 2). PBMCs isolated from 35 healthy controls were stimulated with *Aspergillus* conidia and IL-5 and IFNγ was measured. The homozygote GG genotype of *IL4Rα* (rs1805010) showed a trend towards higher IL-5 production and an increased IL-5/ IFNγ ratio (Figure 6A). The GG genotype in the *IL10* SNP (rs1800896) showed a trend towards higher IFNγ production, however no association was observed with *Aspergillus*-specific IL-5 or IL-5/ IFNγ ratio (Figure 6E). The other polymorphisms in *SFTPA2* (rs17886395 and rs17880349) and *MBL* (rs5030737) did not show an association with IL-5 or IFNγ production (Figure 6B, C and D).

Table 2 | SNPs associated with ABPA

Gene	rs number	SNP position	MAF	function
IL-4Rα (16p12.1- p11.2)	rs1805010	4679 A/G	0.466	missense
SFTPA2 (10q22.3)	rs17886395	1649 C/G	0.206	missense
SFTPA2	rs17880349	1492 C/T	0.461	Intronvariant
IL-10 (1q31-q32)	rs1800896	min 1082 A/G	0.303	near-Gene5
MBL (10q11.2-q21)	rs5030737	868 C/T	0.028	missense

Gene, rs number, SNP position, minor allele frequency (MAF) and resulting defect of gene function of SNPs associated with ABPA are listed

Discussion

In the present study, we investigated the general and *Aspergillus*-specific features of the Th2 response in primary human PBMCs of healthy controls and ABPA patients. Th2 cytokine responses *in vitro* could only be detected when PBMCs were stimulated with *A. fumigatus*, while *Candida,* specific PRR ligands, Gram-positive and Gram-negative bacteria did not induce IL-5 or IL-13 production. Furthermore, no single fungal component induced IL-5 or IL-13 in the same amount as *Aspergillus* conidia, suggesting that a complex of fungal PAMPs present on the cell wall of *Aspergillus* conidia is needed to induce a Th2 response in human PBMCs. The *Aspergillus*-specific IL-5 and IL-13 production was dependent on $CD4^+$ cells, the pattern recognition receptor CR3, and phagocytosis. Moreover, we identified that ABPA patients, in contrast to non-allergic controls, had a significantly elevated Th2/ Th1 ratio induced by *Aspergillus*. Interestingly, not the asthma itself but the *Aspergillus* sensitization

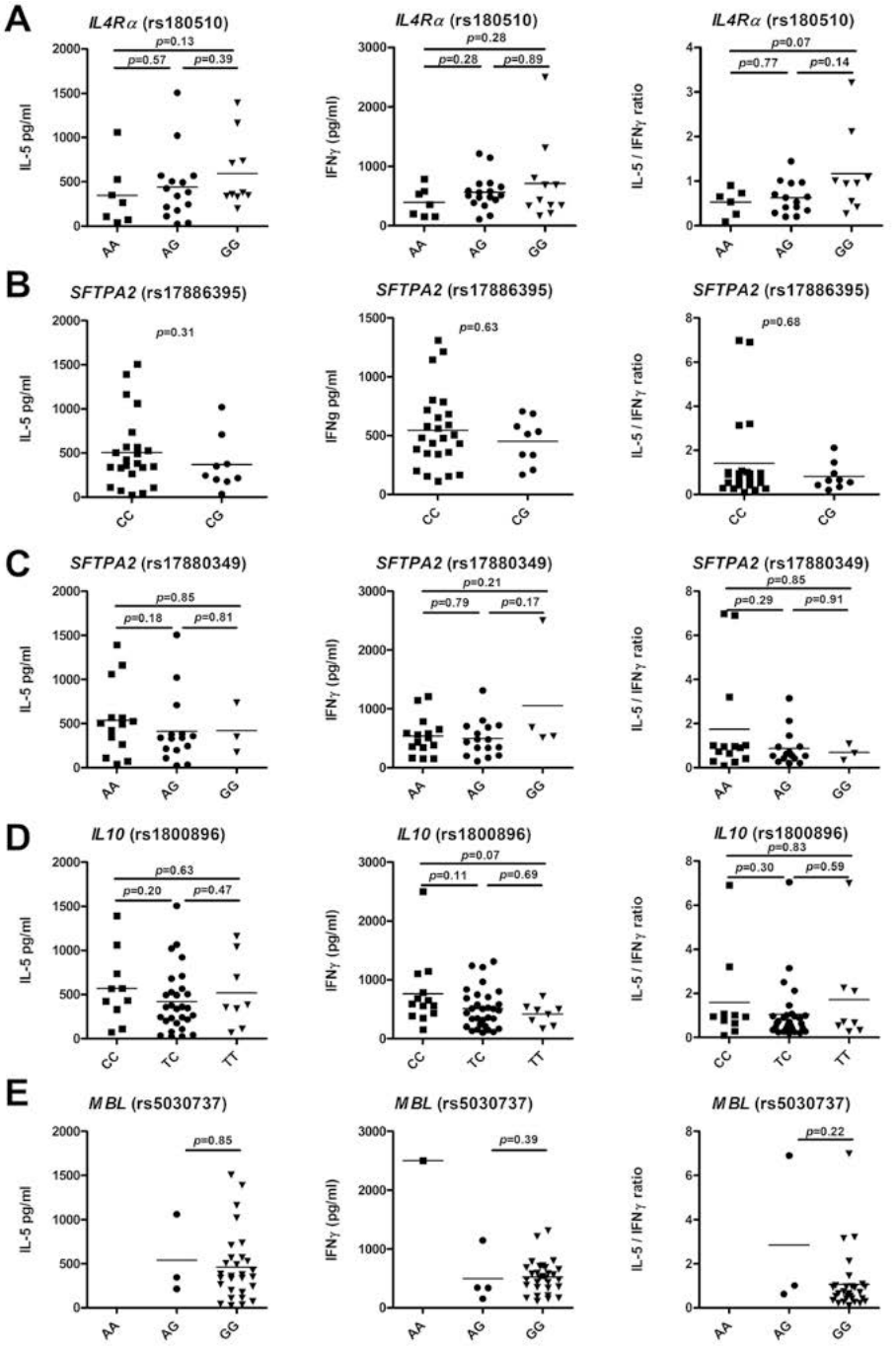

Figure 6 | The role of polymorphisms associated with ABPA on *Aspergillus*-specific Th2/Th1 ratios.
PBMCs isolated from healthy volunteers (n=35) were stimulated with *Aspergillus* conidia and IL-5 and IFNγ were measured in the cell culture supernatant. SNPs were analysed for association with cytokine production. Statistical analysis was performed with the Mann-Whitney-U test.

in asthma patients appears to be responsible for the increased Th2/Th1 ratios observed in ABPA. We suggest that treatment strategies restoring this Th2/Th1 imbalance triggered by *Aspergillus* might be beneficial in the treatment of ABPA. Although anti-TNFα and IL-1Ra had the capacity to decrease *Aspergillus*-induced IL-5 and IL-13 production they also lowered IFNγ production. Therefore, the capacity of recombinant IFNγ to lower IL-5 and IL-13 and to supplement the IFNγ deficiency in ABPA, provides a rationale to use IFNγ as a corticosteroid-sparing treatment option in ABPA.

The observation that the induction of Th2 cytokines in human PBMCs was restricted to *Aspergillus* was striking. In the context of diseases caused by bacteria such as *E. coli*, *S. aureus*, *M. tuberculosis*, *B. burgdorferi* it can be anticipated that they do not induce strong Th2 responses in human PBMCs. However, the observation that no single TLR ligand, NOD2 ligand, or fungal cell wall component, specifically chitin, did not induce IL-5 and IL-13 was unexpected. Chitin has been associated with allergic inflammation in the lung [32,33]. Therefore, it is most likely that chitin in the setting of an inflammatory environment is able to polarize towards Th2 responses, while as a single ligand it is not able to induce a Th2 response.

This study revealed a non-predominant role of TLRs in the induction of *Aspergillus*-specific Th2 cytokines. Blocking of TLR2 did not have a significant impact on IL-5 and IL-13 production induced by *Aspergillus*. In contrast, it has been shown that there is an association between increased TLR2 expression in fatal asthma [34], and polymorphisms in TLR2 are associated with a higher prevalence of asthma [35]. Furthermore, TLR2$^{-/-}$ mice show a deficient Th2 production in a chronic fungal asthma model [13]. The minor but significant contribution of TLR4 in the *Aspergillus*-induced IL-13 induction found in the present study is in line with former studies describing TLR4 in allergic responses against house dust mite in which an endotoxin containing extract signalling via TLR4 [36]. In addition, we identified a redundant role of dectin-1 in the *Aspergillus*-induced Th2 response. Blocking dectin-1 or Syk did not alter IL-5 and IL-13 production, and dectin-1 deficient patients produced Th2 cytokine responses comparable to healthy controls. The dectin-1 ligand β-glucan [17], however has recently been described to play an important role in *Aspergillus*-triggered asthma immunopathology in mice [25]. The role of dectin-1 in the immunopathology of asthma is not clear. It has been suggested that dectin-1 promotes the immunopathology in fungal asthma [25], while another study demonstrated that dectin-1 plays a role in suppressing asthmatic inflammation [37]. Our findings suggest that TLR2 and dectin-1 are not the main pathways driving the *Aspergillus*-specific Th2 response in human mononuclear cells. Whether TLR2 and dectin-1 play a role in *Aspergillus*-induced Th2 responses that are dependent on epithelial cells remains to be elucidated.

Th2 induction by *Aspergillus* was dependent on CR3 (CD11b/CD18). CR3 is a β_2-integrin that contains a lectin-like domain and is expressed on neutrophils and monocytes [27]. CR3 contributes to antifungal host defence by interacting with pentraxin-3 opsonized particles, and by recognizing β-glucan [26]. Furthermore, CR3 is involved in complement driven host responses [38], however the induction of IL-5 and IL-13 was still present in de-complemented serum, suggesting a complement independent contribution of CR3 to the Th2 response. Activation of the complement via the MBL pathway is an alternative route of the complement pathway, and increased MBL serum

concentrations have been associated with ABPA [28]. However, Th2 induction by *Aspergillus* via CR3 signalling was independent of serum MBL, suggesting that MBL is not crucial for CR3-mediated Th2 responses induced by *Aspergillus*. Phagocytosis of non-opsonised particles via signalling together with FcR is also dependent on CR3 [27]. In line with blocking CR3, blocking phagocytosis led to a complete abolishment of Th2 production induced by *Aspergillus*. These data collectively suggest that CR3 is involved in phagocytosis of *Aspergillus* conidia, and that this process is essential for Th2 cytokine production by human PBMCs. Recently, CR3 was described in the induction of the *Aspergillus*-specific pro-inflammatory T-helper cytokines [39], suggesting a more general role for CR3 in modulating T-helper responses.

An important issue to address was the cellular source of the Th2 cytokine in human mononuclear cells induced by *Aspergillus*. We could demonstrate that $CD4^+$, but not $CD19^+$ and $CD56^+$ cells were the predominant source for the *Aspergillus*-induced IL-13 in human PBMCs. This is in line with previous studies showing that $CD4^+$ cells are responsible effector cells in allergic reactions [10], and that $CD19^+$ and $CD56^+$ play a redundant role in an asthmatic mouse model [40]. Although increased levels of $CD8^+$ and IL-13^+ cells have been associated with asthma and bronchial obstruction [41], IL-13 production in human PBMCs was completely dependent on $CD4^+$ cells, suggesting that $CD8^+$ T-cells are not involved in the production of *Aspergillus*-induced IL-13 in human mononuclear cells. We could not demonstrate the cellular source of IL-5 in our setting since IL-5 was not measurable in depletion experiments. Since FACS analysis demonstrated a significant $CD8^+$ population that stained positive for IL-5, we cannot exclude that $CD8^+$ T-cells play a role in *Aspergillus*-induced IL-5 production in human PBMCs.

Several studies have shown an exaggerated Th2 response in ABPA [42-44]. We investigated the *Aspergillus*-specific Th2 response in the context of *Aspergillus*-induced T-helper responses in human PBMCs isolated from ABPA patients. Interestingly, ABPA patients not only show an increased IL-5 and IL-13 production after stimulation with *Aspergillus* conidia, which is in line with previous studies [45], they also have an *Aspergillus*-specific decreased IFNγ production. Since we also observed an increased Th2/Th1 ratio in asthma patients with *Aspergillus*-sensitization, but not in patients with asthma that did not have *Aspergillus*-sensitization, it is most likely that the increased Th2/Th1 ratios in ABPA are due to an *Aspergillus*-specific host response and not due to the underlying host response associated with asthma itself. These observations strengthen the hypothesis that ABPA and asthma with *Aspergillus* sensitization share underlying pathophysiological mechanisms that are distinct from asthma itself, and support the concept that similar treatment approaches could be beneficial in both asthma-sensitized patients and ABPA, such as the treatment with azoles [11].

Aspergillus hyphae have been found in tissue biopsies in ABPA and have been suggested to play a significant role in inflammation in ABPA [46,47]. Furthermore, IgE antibodies directed against Aspf4 and Aspf6 can be observed in ABPA in patients with CF [48,49]. Aspf6 has been shown to be a hyphae-specific protein. Therefore, ABPA patients are exposed to *Aspergillus* hyphae, which contributes to a Th2 response *in vivo*. We observed however that *Aspergillus* hyphae induced much lower amounts of Th2 cytokines in ABPA patients compared to *Aspergillus* conidia, suggesting that fungal cell wall

structures specific for *Aspergillus* conidia, or phagocytosis might play a more prominent role in the induction of Th2 responses in ABPA than previously thought.

Regulatory T-cells (Tregs) are important for controlling allergic immune responses [50]. A recent study has shown, that *Aspergillus*-sensitized asthma patients with high *Aspergillus*-specific Th2 induction had significant decreased levels of *Aspergillus*-specific Tregs, while they were strongly induced in the healthy control group [51]. Also in CF patients with ABPA, Tregs were less frequent than in non-ABPA CF patients [29] suggesting that a deficient Treg response could contribute to the excessive and unbalanced inflammatory response observed in ABPA patients. It is tempting to speculate that the increased *Aspergillus*-specific Th2/Th1 response that we observed in the ABPA patients is due to a deficient Treg response, and that a deficient Tregs response, accompanied by a high Th2 and low Th1 response plays a significant role in the pathophysiology of ABPA.

The characterization of this immunological profile of ABPA patients with an elevated Th2/Th1 ratio provides a read-out for testing potential immunomodulatory treatment options in ABPA. TNFα and IL-1 are interesting targets. TNFα has been shown to be the key cytokine inducing increased IL-17 levels and neutrophilia in the lung in a murine ABPA model [52]. The involvement of the inflammasome and epithelial derived IL-1 cytokines are also described in bronchial asthma in several studies [53,54]. Therefore we tested the TNFα neutralizing biologicals, Etanercept and Adalimumab, and the IL-1 pathway blocking biological IL-1Ra (anakinra). All biologicals decreased IL-5 and IL-13 production, but also IFNγ production, therefore resulting in unaltered IL-13/IFNγ and IL-5/IFNγ ratios. In addition we investigated recombinant IFNγ, which decreased *Aspergillus*-specific IL-5 and IL-13 production.
The co-stimulatory molecule OX40L is involved in the initiation and maintenance of allergic responses, mainly driven by TSLP activated dendritic cells [55]. Clinical studies that have been performed to elucidate the role of OX40L as a possible therapeutic target in asthma and have showed that blocking OX40L results in a decrease of serum IgE and eosinophils in asthma [56]. OX40L was also involved in the *Aspergillus*-induced Th2 response, which is in line with former studies showing OX40L expressed on a variety of cells like activated T-cells, NK cells, B-cells and monocytes [57]. We observed a low expression of OX40L in PBMCs that was not significantly modulated by TNFα or IL-1. Although it is reported that TNFα can increase OX40L expression in smooth muscle cells, significantly higher in asthmatic than non-asthmatic patients [30] and TNFα can up-regulate OX40L promoter-activity [31], this mechanism appears to play a more prominent role in DC-driven Th2 responses [58].

Taking the shifted *Aspergillus*-induced Th1/Th2 ratio into account, restoring the increased Th1/Th2-ratio might be an interesting alternative for target-specific immunomodulatory therapy in ABPA. Previous studies have shown that neutralizing IL-13 has beneficial effects in an asthmatic mouse model [59] by dampening the IL-13 driven allergic airway inflammation. Similarly, blocking IL-4 has been tried to attenuate allergic airway responsiveness, however the effect was not as strong and long-lasting as blocking IL-13 [59]. Biologicals targeting IL-4, IL-5 and IL-13 have been shown to have some efficacy in patients with high Th2 cytokine levels, however optimal biomarkers to identify responders and non-responders are still lacking [60]. There is only little clinical evidence for immunomodulatory treatment with biologicals in ABPA. Omalizumab has been successfully used

in a few cases of ABPA with and without CF resulting in a corticosteroid-sparing treatment [61-63]. The data of this study suggests that IFNγ might be an adequate immunomodulatory therapeutic option for ABPA, since it has the capacity to restore the increased Th1/Th2-ratio. Next to lowering the Th2 response it also supplements the relative *Aspergillus*-specific IFNγ deficiency. Recombinant IFNγ has been used with success in patients with chronic granulomatous disease to prevent *Aspergillus* infection [64] and there are several case reports that describe the beneficial effect of IFNγ treatment in *Aspergillus* infection [65]. Thus, IFNγ might contribute to disease control in ABPA by having a beneficial effect on clearing the fungal burden thereby decreasing the trigger of the allergic inflammatory reaction, and by restoring the increased Th2/Th1 ratio.

Single nucleotide polymorphisms (SNPs) in the *IL10*, *IL4Ra*, *MBL*, and *SFTPA2* genes have been associated with an increased susceptibility to ABPA [19]. We therefore investigated whether these genetic variants could modulate the *Aspergillus*-induced Th1 or Th2 response. Variants in the *MBL* [66] and *SFTPA2* [67] genes did not lead to different *Aspergillus*-specific Th2 and Th1 responses. In contrast, we could identify a trend between the *IL4Ra* [68] GG genotype with higher *Aspergillus*-specific IL-5 production and *Aspergillus*-specific Th2/Th1 ratio. We observed a trend in the *IL10* (rs1800896) GG genotype towards higher IFNγ production. Previous studies have identified this genotype to be associated with *Aspergillus* colonization and ABPA in CF patients [69].

In conclusion, *Aspergillus* conidia are unique in triggering Th2 responses in human PBMCs, and we provide evidence that CR3 and phagocytosis play a predominant role in *Aspergillus*-induced Th2 responses. Moreover, we identified that patients with ABPA have an increased *Aspergillus*-specific Th2/Th1 ratio, and the data in this study together with the clinical experience and safety of IFNγ treatment provide a rationale for exploring IFNγ in a clinical trial in patients with ABPA.

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Supplementary material

Table S1 | Demographic table of asthma patients

No.	age	Sex	*Asp*-IgE (U/mL)	*Asp*-IgG (mg/mL)	total IgE (U/mL)
Asp-asthma 1	61	M	>5	neg	1913
Asp-asthma 2	76	M	11,2	62,1	570
Asp-asthma 3	44	M	>5	3,76	52
asthma 1	44	F	<0,35	n.a.	79
asthma 2	23	F	<0,35	36,2	212
asthma 3	47	F	<0,35	4,11	182
asthma 4	47	F	<0,35	43,1	95

Age, sex, total IgE (U/mL), *Aspergillus*-specific IgE (*Asp* IgE)(U/mL) and *Asp*ergillus-specific IgG (*Asp*-IgG) (mg/mL) of the asthmatic control groups enrolled in this study are listed.

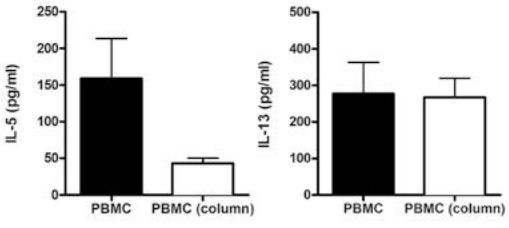


Figure S1 | Th2 response against *Aspergillus* conidia before and after depletion column.
PBMCs were either directly stimulated or mock treated by running unlabelled cells of a cell depletion column. PBMCs ($2.5x10^6$/mL, n=4 donors) were stimulated with *Aspergillus* conidia and IL-5 and IL-13 were measured in the cell culture supernatant by ELISA.

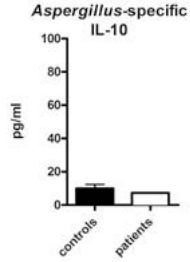
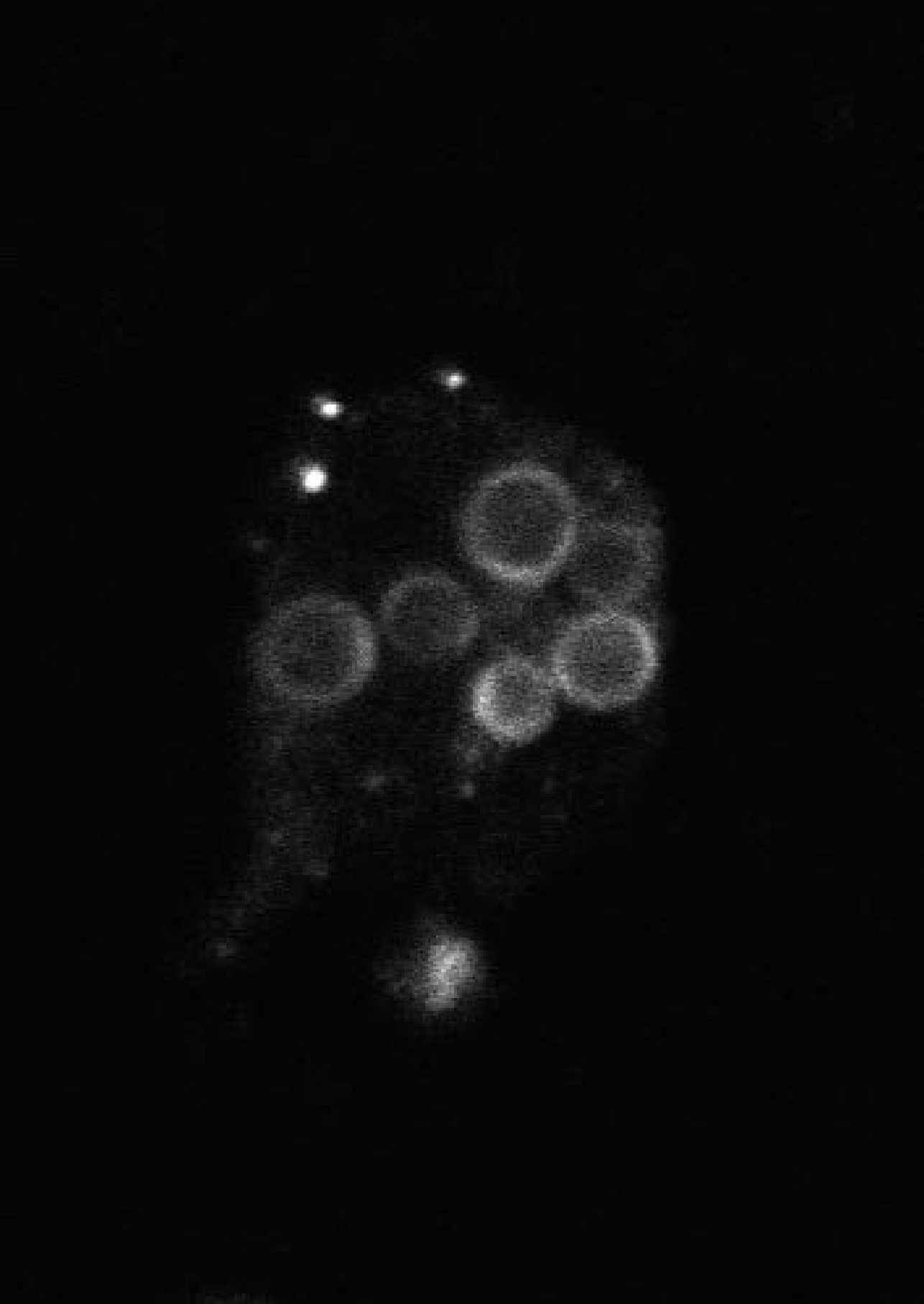


Figure S2 | *Aspergillus*-induced IL-10 in PBMCs of ABPA patients and healthy controls
PBMCs (n=9 ABPA patients or healthy controls) were stimulated with *Aspergillus* conidia for 7 days and IL-10 was measured in the cell culture supernatant by ELISA.



Chapter 6

The absence of NOD1 protects immunosuppressed mice against invasive aspergillosis through enhanced fungal killing

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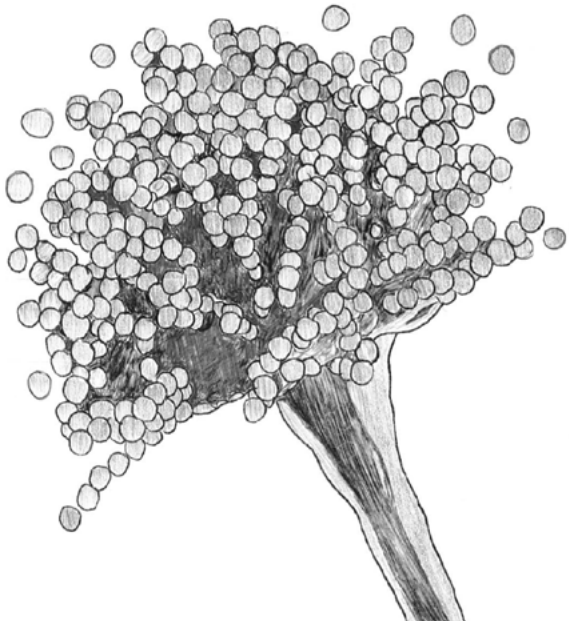

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Submitted

Abstract

Invasive aspergillosis remains one of the most severe complications in immunocompromised patients. Recognition of *Aspergillus* by pattern recognition receptors (PRRs) is crucial for initiating host defence to protect against invasive aspergillosis. The intracellular pattern recognition receptors nucleotide-binding oligomerization domain (NOD)1 and NOD2 are an important class of PRRs that are unexplored in invasive aspergillosis. Studies have suggested a role for NOD2 in the recognition of *Aspergillus*, while nothing is known about NOD1. Here we systematically explored the role of NOD1 and NOD2 in the host defence against invasive aspergillosis. Strikingly, immunosuppressed (cyclophosphamide) NOD1 and NOD2 deficient mice were protected against invasive aspergillosis, which was reflected by significant decreased mortality. NOD2 deficient mice however were clinically more ill compared to wild type mice and had increased inflammatory responses, while NOD1 deficient mice rapidly cleared the infection. In contrast to NOD2 deficient cells, NOD1 deficient cells are more efficient at inducing cytokines in response to *Aspergillus* and at killing *A. fumigatus*. In healthy volunteers, SNPs in NOD1 were associated with an increased Th17 response while SNPs in NOD2, like NOD2 deficiency in patients with Crohn's disease were associated with decreased cytokine responses to *A. fumigatus*, suggesting that NOD1 and NOD2 indeed modulate human host defence against *Aspergillus*. Collectively, our data demonstrates that the NOD receptors are involved in the host defence against *Aspergillus*. In particular we have identified that NOD1 deficiency can be protective in an immunosuppressed host with invasive aspergillosis, which makes NOD1 a potential treatment target to explore in invasive aspergillosis.

Introduction

Humans are ubiquitously exposed to *Aspergillus,* but only the immunocompromised host is at risk of developing potentially life-threatening infections. Invasive aspergillosis (IA) is one of the most severe complications in immunocompromised patients with high mortality [1]. Knowledge of the antifungal host response that is required for clearance of the infection is crucial for the development of new immunomodulatory therapies. Although the pattern recognition receptor (PRR) pathways that play a role in recognition of *Aspergillus* and activation of the immune system are generally known [2,3], still new pathways are being discovered to be involved in *Aspergillus* recognition. An interesting observation is that a patient with Crohn's disease, deficient for the intracellular nucleotide-binding oligomerization domain (NOD)2 receptor developed invasive aspergillosis following anti-TNFα treatment [4]. Recently, the nucleotide-binding oligomerization domain (NOD)2 receptor was suggested to play a role in the recognition of *Aspergillus*-derived chitin [5]. Furthermore, exposure to *Aspergillus* was found to increase NOD2 expression in the lungs of mice, THP1 cells and A549 cells. In addition, co-stimulation of these cell lines with *A. fumigatus* and the NOD2 ligand GlcNAc-MurNAc dipeptide (MDP) synergistically induced tumour necrosis factor (TNF)α production [6], and NFκB activation in RAW macrophages was found to be dependent on NOD2 [7]. An additional role for NOD2 has been suggested in the host defence against fungal keratitis. Stimulation of the human corneal epithelial cell line (THCE) with *Aspergillus* was shown to increase NOD2 expression, besides IL-8 and TNFα production was found to be partly NOD2 dependent [8]. NOD2 belongs to the NACHT-LRR receptor (NLR) family and is closely related to NOD1. Both receptors are involved in the recognition of peptidoglycan-derived moieties and in the induction of pro-inflammatory [9-13]. After activation by their ligands, NOD1 and NOD2 recruit their downstream signalling adaptor RICK which subsequently activates NFκB [14]. Although, previous studies have focused on the role of NOD2 in the host response to *Aspergillus*, the role of NOD1 has not been thoroughly investigated. Furthermore, the effect of NOD receptor deficiency on susceptibility to IA remains unexplored. Therefore, in the present study we investigated whether deficiency of NOD1 and NOD2 influences susceptibility to invasive aspergillosis and whether it affects the immune response to *Aspergillus*.

Experimental procedures

***In vivo* experiments |** Mice for *in vivo* experiments were supplied by the breeding center R. Janvier (Le Genest Saint-Isle, France). Mice were cared for in accordance with Institut Pasteur guidelines, in compliance with European animal welfare regulation. Upon arrival, animals were placed in isolated ventilated cages.

For the survival experiment in an immunosuppressed background C57/BL6 wild-type(5m/6f), NOD1$^{-/-}$ (6m/6f) and NOD2$^{-/-}$ (7m/2f) mice (28 to 31g, 10 weeks old) were used. Mice were immunosuppressed at day 4 and day 1 before infection by intraperitoneal injection of 200µL cyclophosphamide (Sigma Aldrich) at 4 mg/mL. At the day of infection mice were anaesthetized by intramuscular injection (150µL) of ketamine (10mg/mL) and xylasine (10mg/mL) hair was shaved

from the ventral lung area and subsequently mice were inoculated intranasally with $5x10^4$ luciferase expressing *A. fumigatus* 2/7/1 conidia [15] in 25 µL PBS.

In all experiments survival and weight was monitored during the course of infection. Bioluminescence imaging was acquired at day 1 post infection (pi) and was continued on day 2, 3, 6 and 8 pi. Images were acquired using an IVIS 100 system (PerkinElmer, Waltham, MA) as previously described [16].

For immunological assessment female C57/BL6 mice (19 to 22g, 8 weeks old) were used. They received similar immunosuppression regimen and were similarly infected as the mice for survival. Weight and bioluminescence were monitored daily during the course of infection. At day 3 the mice were euthanized. Serum and BAL were collected and lung homogenates were obtained following disruption in saline using the Retsch Mixer Mill 301 homogenizer. Cytokines concentrations in lung supernatants, BAL, and plasma were determined by ELISA as specified by the manufacturer (DuoSet; R&D Systems)

Histology | Sinuses and lungs were removed and immediately fixed in 10% neutral-buffered formalin. After fixation, sinuses were decalcified for 1 month, using a chelating agent (ethylenediaminetetracetic acid – EDTA) in order to allow routine processing to paraffin while preserving high quality morphology. Sinus and lung samples were then embedded in paraffin, and cut into 4 µm thick sections. Serial sections were stained with hematoxylin and eosin (HE) for description or histological lesions and Grocott's methenamine silver for fungal detection.

***Ex vivo* stimulation of WT, NOD1$^{-/-}$ and NOD2$^{-/-}$ murine splenocytes |** Wild-type, NOD1$^{-/-}$ and NOD2$^{-/-}$ C57Bl/6 mice were bred and maintained in the St. Jude Children's Research Hospital, Memphis, TN, USA. Animal studies were conducted under protocols approved by St. Jude Children's Research Hospital Committee on Use and Care of Animals. Spleens were homogenized in 0,4µM cell strainer (BD) and the cell number was adjusted to $1x10^7$/mL. The cell suspensions (500µL /well) were placed in a 24 well plates (corning) and incubated with culture medium or *Aspergillus* conidia for 2 or 5 days at 37°C and 5% CO_2. After stimulation culture supernatants were collected and stored at -20°C until cytokine measurement.

Killing of *Aspergillus* by bonemarrow derived macrophages | Bone marrow from mice (age between 8–20 weeks) was flushed out after dissecting mouse legs. Differentiation into macrophages (BMDMs) occurred in 5 days at 37°C (5% CO_2) in Iscove's modified Dulbecco's medium (IMDM) supplemented with 30% of L929 supernatant containing 10% foetal bovine serum (heat-inactivated, Invitrogen), 100 U / mL penicillin and 100 mg/mL streptomycin. Following differentiation the BMDMs ($1x10^5$) were exposed to *Aspergillus* conidia ($2x10^6$) in a final volume of 200µL. After 24 hours at 37°C the cells were washed in water and plated in serial dilution on sabouroud agar plates. CFUs were counted after 24 hours at 37°C.

ROS induction | The induction of reactive oxygen species were measured by oxidation luminol (5-amino-2,3,dihydro-1,4-phtalazinedione). PBMCs ($5x10^5$) or BMDMs ($1x10^5$) were resuspended in HBSS and put in dark 96 well plates. Cells were exposed to HBSS, *A. fumigatus* conidia ($1x10^7$/mL), *A. fumigatus* germs ($1x10^7$/mL) or Zymosan (150µg/mL) and immediately 20µL of 1mM luminol was added. Chemiluminescence was measured in BioTek Synergy HTreader at 37°C for every minute during one hour.

Healthy controls and patients | Venous blood samples from healthy controls and patients were obtained after written informed consent and were analysed for polymorphisms in the NOD1 and NOD2 genes. Six patients with Crohn's disease that were homozygous for the Leu1007fsinsC polymorphism were included for studying NOD2 deficiency after informed consent was obtained.

***Aspergillus fumigatus* |** A clinical isolate of *Aspergillus fumigatus* V05-27, which has been characterized previously [17] was used for all *ex vivo* and *in vitro* stimulations. Conidia and Hyphae were prepared and heat-inactivated (HI) as previously described.[18] A concentration of $1x10^7$/mL was used in the experiments, unless otherwise indicated. *Aspergillus* conidia were FITC labelled as previously described [19].

PBMC isolation and stimulation | Venous blood was drawn into 10 ml EDTA tubes. PBMCs were isolated as previously described (23). Briefly, blood was diluted in PBS (1:1) and fractions were separated by Ficoll (Ficoll-Paque Plus, GE healthcare, Zeist, The Netherlands) density gradient centrifugation. Cells were washed twice with PBS and resuspended in RPMI-1640^+ (RPMI1640 dutch modification supplemented with 10 µg/mL gentamycin, 10 mM L-glutamine and 10 mM pyruvate; Gibco, Invitrogen, Breda, The Netherlands).
PBMCs were plated in 96 well roundbottom plates (Corning, NY, USA) at a final concentration of $2,5x10^6$ cells/mL and in a total volume of 200 µL. The individuals in the NOD1 and NOD2 polymorphism cohort were stimulated with medium (negative control) or live *Aspergillus* at a final concentration of $1x10^7$/mL. The NOD2 deficient patients were stimulated in the presence of 10% serum with medium, live *A. fumigatus* conidia ($1x10^7$/mL) , HI conidia ($1x10^7$/mL) or HI hyphae (derived from $1x10^7$/mL conidia). After stimulation culture supernatants were collected and stored at -20°C until cytokine measurement.

Intracellular IL-17, IL-22, and IFNγ flowcytometry | Following 7 days stimulation with *A. fumigatus* conidia, PBMCs were stimulated 4-6 hours with PMA (50 ng/mL) (Sigma-Aldrich), ionomycin (1 µg/mL) (Sigma-Aldrich) and Golgiplug (BD Biosciences, Breda, the Netherlands) according to the protocol supplied by the manufacturer. Cells were stained extracellular using PeCy7-conjugated anti-CD4 (BD Biosciences) antibody. Subsequently the cells were fixed and permeabilized with Cytofix/Cytoperm solution (eBioscience) according to the protocol supplied by the manufacturer. Following permeabilization the cells were stained intracellular with $Alexa^{647}$-conjugated anti-

IL-17 (BD Biosciences), PE-conjugated anti-IL-22 (R&D systems) and FITC-conjugated anti-IFNγ (eBioscience) according to the protocol supplied by the manufacturers. The cells were measured on a FC500 flowcytometer (Beckman Coulter) and the data were analyzed using CXP analysis software v2.2 (Beckman Coulter).

Cytokine measurements | The cytokine levels were measured using commercially available ELISA assays according to the protocol supplied by the manufacturer. IL-1β, TNFα, IL-17 and IL-22 assays were from R&D systems (Minneapolis MN) and Interferon(IFN)γ was from Sanquin (Amsterdam, The Netherlands). Mouse IL-1β, TNFα, IL-6, KC, IL-17, IL-22, and IFNγ in splenocyte stimulations were measured using the luminex multiplex platform (Millipore, Billerica, MA). For the *in vivo* experiments mouse IL-1β, TNFα, IL-6, KC and G-CSF were measured using commercially available ELISA assays from R&D systems according to the protocol supplied by the manufacturer.

Statistical analysis | Data are presented as mean ± standard error of the mean (SEM), unless otherwise indicated. Unless otherwise indicated the Mann-Withney U test was used to determine statistical significant differences between experimental groups with $p<0.05$ = *, $p<0.01$ = ** and $p<0.001$ = ***. All data were analysed using Graphpad Prism v5.0.

Results

NOD1 deficient immunosuppressed mice are protected against invasive aspergillosis

We investigated whether NOD1 and NOD2 deficiency influences the susceptibility of mice to lethal *Aspergillus* infection in imunnocompromised mice. Several survival experiment were performed where wild-type C57Bl6 with NOD1$^{-/-}$ and NOD2$^{-/-}$ mice were immunosuppressed with cyclophosphamide and subsequently infected with the bioluminescent *Aspergillus* strain 2/7/1 [15]. In contrast, to wild-type, NOD1 and NOD2 deficient mice were protected against lethal infection (Figure 1A). from the NOD1$^{-/-}$ mice 9 out of 12 mice survived and started to recover their weight from day 4 post infection, whereas wild-type and NOD2$^{-/-}$ continued to decline in weight (Figure 1B). Wild-type mice continued to decline in weight and 10 out of 11 mice did not survive the infection whereas 8 out of 9 NOD2$^{-/-}$ mice survived the infection and slowly started to recover from day 6 pi onwards. Strikingly, there was a significant difference in weight loss between NOD1$^{-/-}$ mice and NOD2$^{-/-}$ mice (Figure 1B), which decreased in weight up to day six but eventually recover from the infection. In addition, the NOD2$^{-/-}$ mice demonstrated severe symptoms such as head tilting and circling, symptoms that have been described in the *in vivo* aspergillosis mouse model [20]. Bioluminescence imaging of the luciferase expressing *Aspergillus* in the mice revealed that NOD1$^{-/-}$ and NOD2$^{-/-}$ mice rapidly cleared the infection whereas wild-type developed much higher fungal burdens as indicated by the luminescence signal (Figure 1C).

NOD1$^{-/-}$ mice do not develop inflammation following *Aspergillus* infection

To evaluate differences in tissue damage and inflammation in the mice another experiment was performed where mice were sacrificed at the peak of infection at day three post infection. All groups demonstrated similar weight loss until day 3 post infection (Figure 2A). However, bioluminescence imaging of the luciferase expressing *Aspergillus* revealed that wild-type mice develop high fungal burden within the lungs and the sinuses whereas NOD1$^{-/-}$ mice rapidly clear the fungus from the lungs. In contrast, NOD2$^{-/-}$ mice keep a stable fungal burden in the lungs following the course of

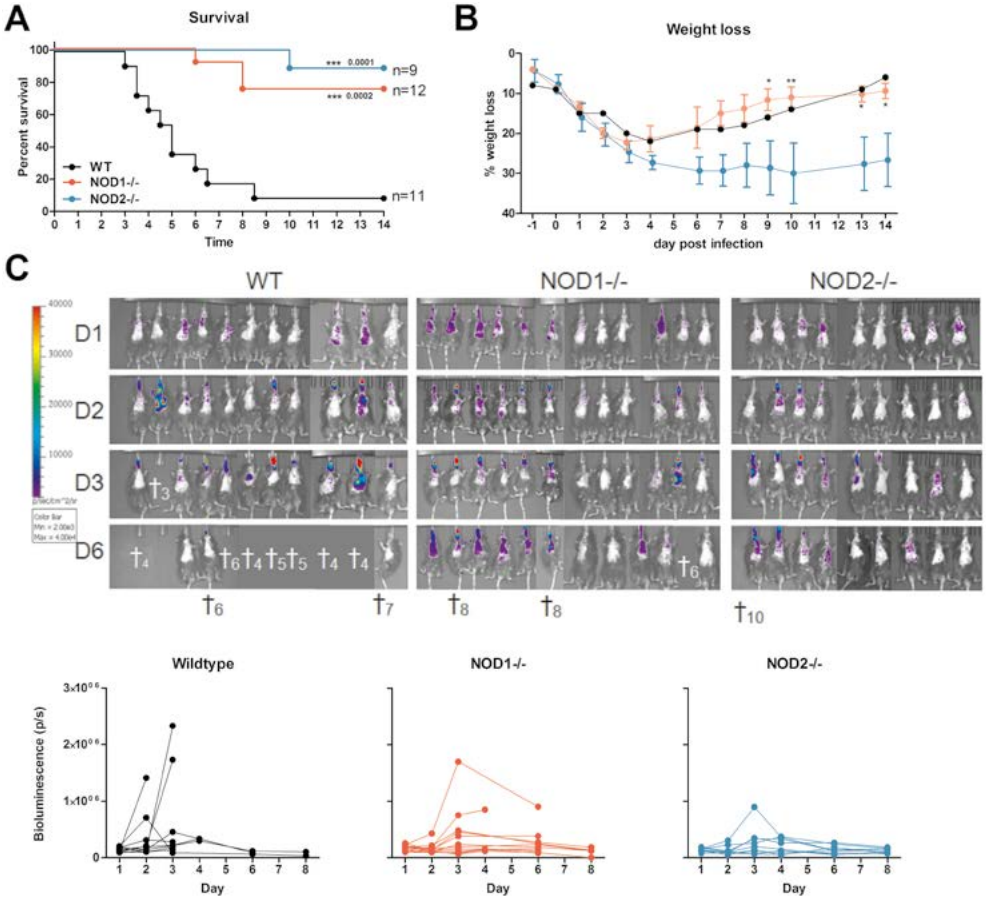

Figure 1 | NOD1$^{-/-}$ and NOD2$^{-/-}$ survive lethal *A. fumigatus* challenge

(A) Kaplan-Meier survival curve of cyclophosphamide immunosuppressed WT (n=11), NOD1$^{-/-}$ (n=12) and NOD2$^{-/-}$ (n=9) mice infected intranassaly with 5x10^4 conidia. P values of the Kaplan-Meier curve were determined with the use of the log-rank test. Data represent the cumulative data of four separate experiments (5vs.6vs.4; 3vs.6vs.0; 3vs.0vs.2; 3vs.0vs.3) . (B) Representative graph of percentage weight loss of surviving mice in one of the experiments where survival of WT (n=5; 4 died; n=1 shown), NOD1$^{-/-}$ (n=6; 1 died; n=5 shown) and NOD2$^{-/-}$ (n=4; 1 died; n=3 shown) mice was compared. Timepoints were compared for significance by two-way ANOVA. (C) Bioluminescence signal of luciferase positive *A. fumigatus* within the mice at day 1, 2, 3, 4, 6 and 8 post infection measured (nd = bioluminescence imaging was not performed; † = mouse died). The graphs represent the signal of the thoracic and sinus area (each mouse is shown individually).

infection that is higher than in NOD1$^{-/-}$ mice (Figure 2B and C). To assess systemic inflammation, cytokines were measured in the plasma. No difference in IL-1β concentration was observed between wild-type and NOD1$^{-/-}$ mice, whereas NOD2$^{-/-}$ mice demonstrate a trend towards elevated IL-1β levels. IL-6 levels were lower in the plasma of NOD1 and NOD2 deficient mice but this difference was not significant. Interestingly, NOD2$^{-/-}$ mice demonstrate significantly higher KC plasma levels than NOD1$^{-/-}$ mice, but no significant difference compared to wild-type was observed (Figure 2D). Broncheoalveolar lavages (BALs) and lung homogenates at day 3 post infection were obtained to investigate inflammation in the lung. NOD1$^{-/-}$ mice demonstrated no induction of KC and IL-6 whereas NOD2$^{-/-}$ mice demonstrated increased IL-6 and KC responses compared to NOD1$^{-/-}$ mice (Figure 2E). No significant differences were observed in the lung homogenates except a trend towards increased TNFα levels when comparing WT and NOD1$^{-/-}$ mice (Figure 2F).

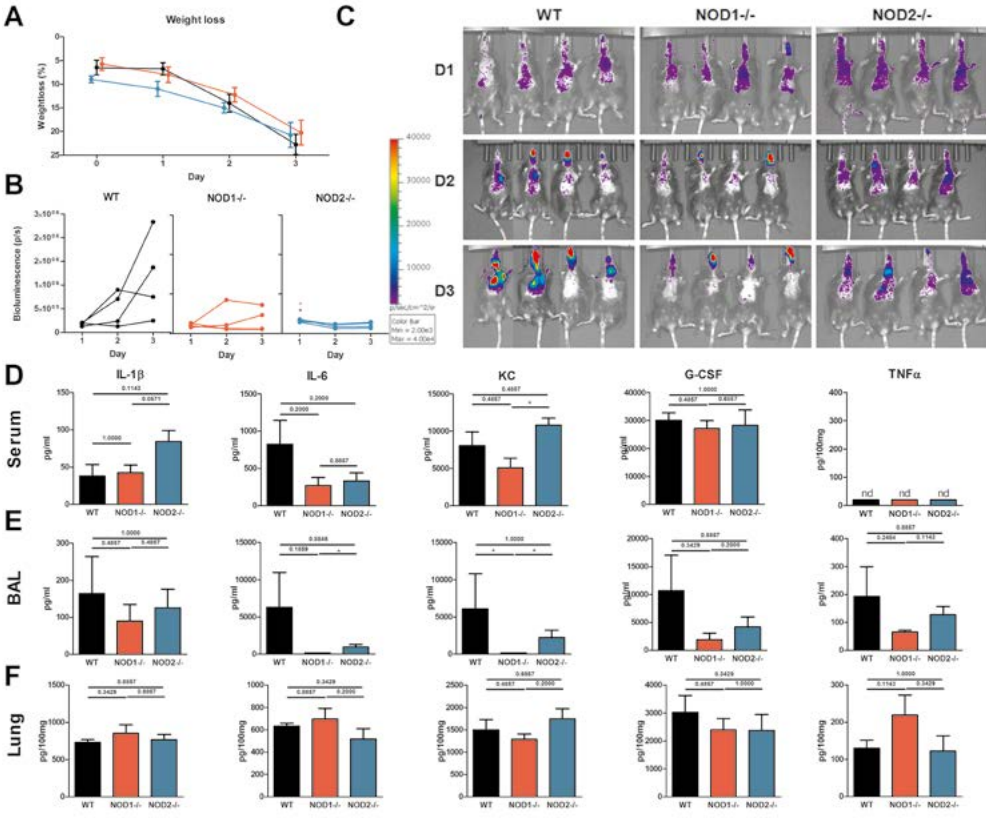

Figure 2 | Inflammation during *Aspergillus* challenge in WT, NOD1$^{-/-}$ and NOD2$^{-/-}$ mice
(A) Percentage weightloss following cyclophosphamide immunosuppression and intranasal *Aspergillus* infection (5x10^4/mouse) in WT (n=4; f), NOD1$^{-/-}$ (n=4; f), and NOD2$^{-/-}$ (n=4; f) mice. (B, C) Luminescence signal at day 1 to 3 post infection from the luminescent *Aspergillus* within the mice. (D, E, F) IL-1β, IL-6, KC, G-CSF and TNFα levels in (D) serum, (E) broncheo alveolar lavage (BAL), and (F) lung homogenates, measured at day 3 post infection.

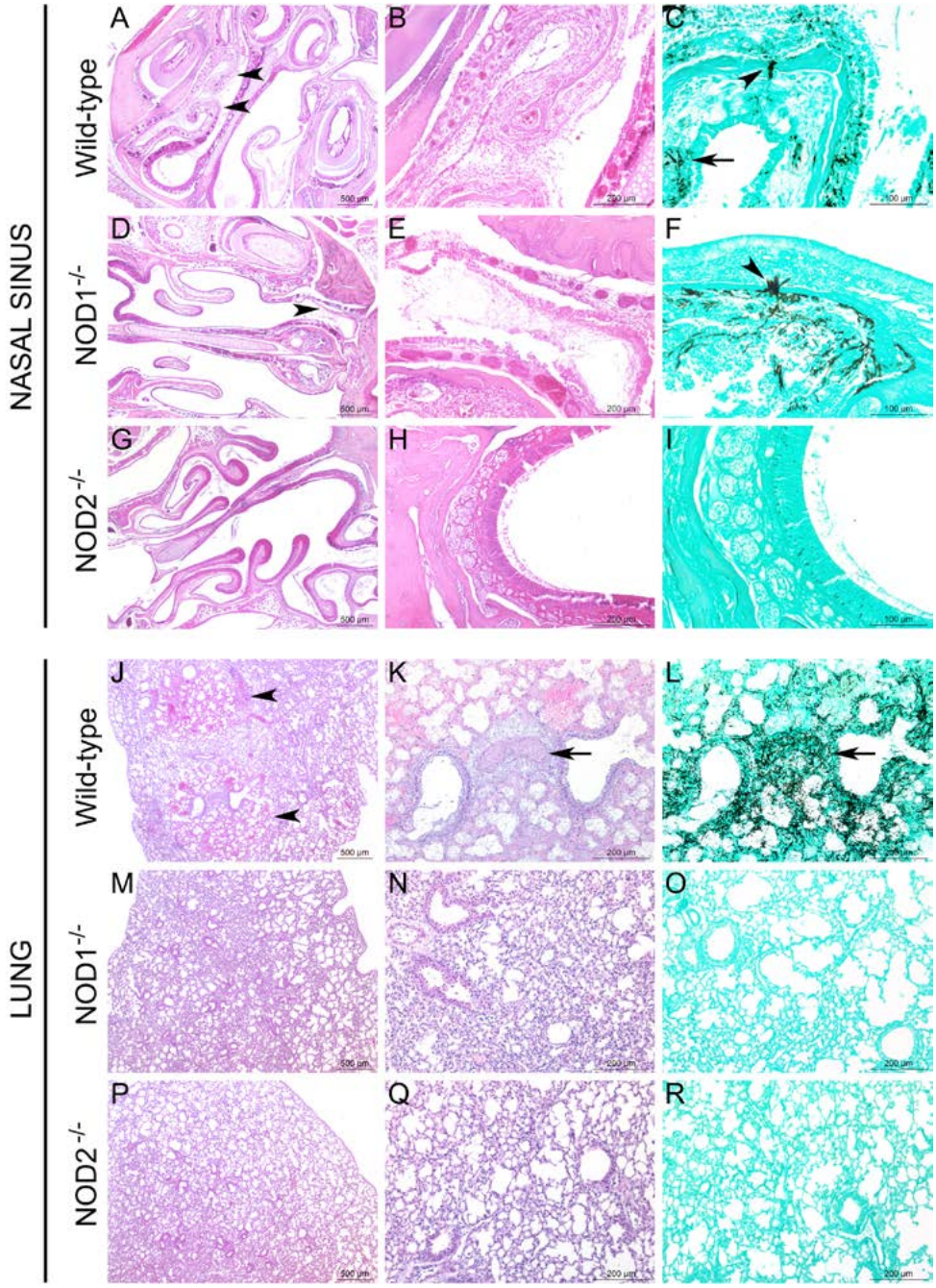

Figure 3 | histolology of sinuses and lungs

(A-I) Histology of Nasal Sinuses of wild-type, NOD1$^{-/-}$ and NOD2$^{-/-}$ mice at day 3 post infection, stained by HE staining at (A,D,G) 2x and (B,E,H) 10x magnification and (C,F,I) Grocott's Methenamine Silver staining at 20x magnification. (A-C) Wild-type mice: (A) Unilateral extensive sinusitis (arrowheads), characterised by (B) destruction of the mucosa, and (C) a high density of hyphae (arrow), multifocally invading bone (arrowhead).

(Figure 3 continued) (D-F) NOD1 deficient mice: (D) Focal unilateral sinusitis (arrowhead), with (E) destruction of the mucosa, less severe than lesions observed in sinuses of wildtype mice, and (F) hyphae, focally invading bone (in only 1/4 mouse) (arrowhead). (G-I) NOD2 deficient mice: (G,H) No histological lesions and (I) no hyphae detected in the tissue (only 1/4 mouse displayed a minimal lesion with very few hyphae; data not shown).
(J-R) Histology of Lungs of wild-type, NOD1$^{-/-}$ and NOD2$^{-/-}$ mice at day 3 post infection, stained by HE staining at (J,M,P) 2x and (K,N,Q) 10x magnification and (L,O,R) Grocott's Methenamine Silver staining at 10x magnification. (J-L) Wild-type mice: (J) Multifocal lesions (arrowheads), (K) centred on bronchi/bronchioles and secondary extending to blood vessels (arrow) and alveoli, characterised by necrosis of lung parenchyma and (L) marked invasion by a high density of hyphae (arrow: blood vessel infiltrated by hyphae). (M-N, P-Q) NOD1 and NOD2 deficient mice: No histological lesion and (O,R) no hyphae detected in the pulmonary tissue.

Histology of lungs and nasal sinuses

Histopathological analysis was carried out at day 3 post infection to asses inflammation and pathological damage lungs and sinuses. In nasal sinuses, wild-type mice displayed invasive, unilateral, subacute sinusitis, characterised by destruction of the mucosa in three out of four mice, secondarily extending to bone in two out of four mice, with a high number of hyphae (Figure 3A-C). NOD1 deficient mice displayed less severe lesions with bone invasion only in one out of four mice (Figure 3D-F). In contrast, NOD2 deficient mice did not display any nasal sinus lesions (Figure 3G-I). In the lung, lesions were only observed in the wild-type group. Multifocal necrotic lesions were identified, centred on bronchi/bronchioles, but secondarily extending to blood vessels and alveoli, characterised by destruction of the mucosa and marked necrosis of epithelial cells, destruction of blood vessel walls with multifocal thrombi, and high density of hyphae. (Figure 3J-L). In contrast, no histological lesions were observed in the lungs of NOD1 and NOD2 deficient mice (Figure 3M-R), except for a very small focus of infection in one out of four NOD2-deficient mice, containing only a few hyphae (data not shown).

NOD receptor deficiency differentially modulates *ex vivo* cytokine responses in murine cells

To explain the observed protection of NOD1 and NOD2 deficiency it was investigated how NOD1 and NOD2 influence the cytokine response against *A. fumigatus*. Splenocytes were isolated from naive NOD1$^{-/-}$, NOD2$^{-/-}$, and WT C57Bl/6 mice, and stimulated with *Aspergillus* and cytokine responses were compared (Figure 4A and B). Although the cytokine responses induced were generally low, a trend towards decreased TNFα, IL-6 (Figure 4A) responses could be observed with NOD2$^{-/-}$ splenocytes, whereas IL-17, IL-22 and IFNγ were undetectable (ud) in the culture supernatants (Figure 4B). In contrast, splenocytes of NOD1$^{-/-}$ mice induced significantly more TNFα and KC in response to *Aspergillus* (Figure 4A). The T-cell cytokines IL-17 and IFNγ were undetectable (ud) and IL-22 was very poorly induced in the culture supernatants by wild-type mice, but these cytokines were significantly elevated in NOD1$^{-/-}$ splenocytes (Figure 4B). In addition to cytokine responses of splenocytes we investigated cytokine responses in bone marrow derived macrophages (BMDMs). Similar to splenocytes, BMDMs from NOD1$^{-/-}$ mice demonstrated significantly increased cytokine responses, with high production of IL-6 and KC, while the responses of NOD2$^{-/-}$ BMDMs were similar to wild-type (Figure 4C).

In addition to cytokine responses, the capacity to kill *A. fumigatus* conidia by wild-type, NOD1$^{-/-}$ and NOD2$^{-/-}$ BMDMs was investigated. NOD1$^{-/-}$ BMDMs where more efficient in killing *Aspergillus* conidia than wild-type BMDMs (Figure 4D) reflected by reduced outgrowth of hyphae (Figure 4E). NOD2$^{-/-}$ BMDMs did not demonstrate a difference in killing *Aspergillus* compared to wild-type BMDMs. In addition, the capacity to generate an oxidative burst was investigated. Within the timeframe that luminescence was measured, the area under the curve was calculated to illustrate the quantitative difference in ROS release (Figure 4G and H). with zymosan (Figure 4G) or *Aspergillus* (Figure 4H). Zymosan-induced ROS release by BMDMs was significantly higher in NOD1$^{-/-}$ BMDMs compared to wild-type BMDMs (Figure 4F and G). Although serum opsonised *Aspergillus* dormant conidia and germinated conidia induced ROS at much lower levels than zymosan, NOD1$^{-/-}$. BMDMs again induced more ROS. By contrast, zymosan and *Aspergillus*-induced ROS induction by NOD2$^{-/-}$ BMDMs was significantly lower than wild-type BMDMs.

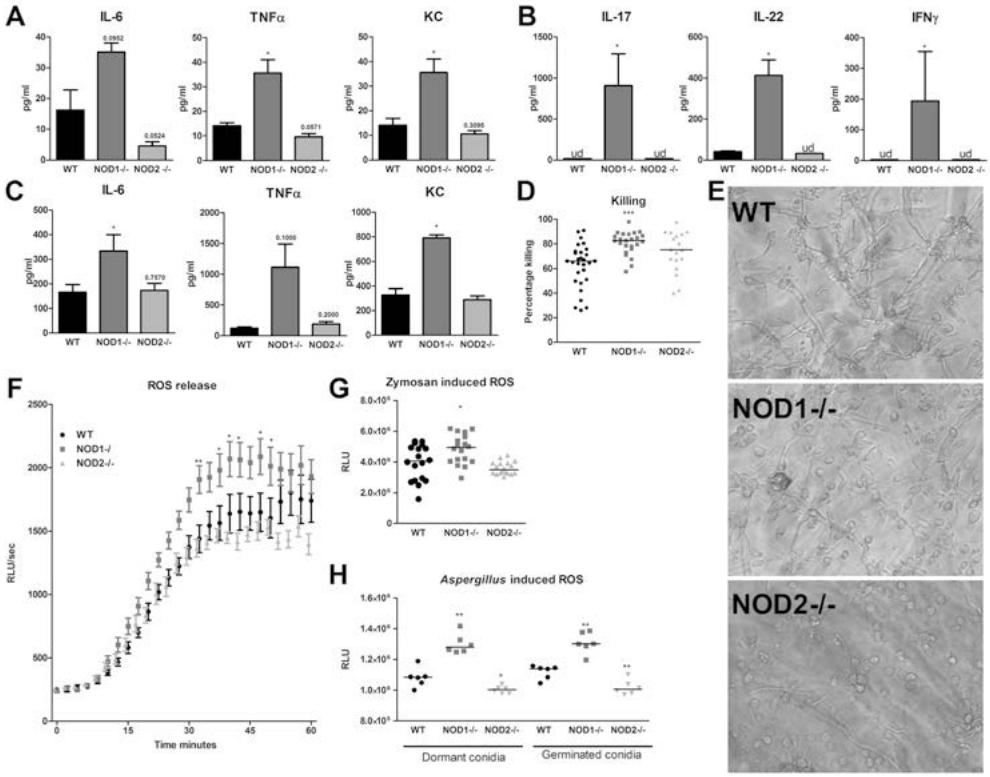

Figure 4 | *in vitro* responses of murine NOD1 and NOD2 deficient splenocytes and fungal killing by BMDMs (A) IL-6, TNFα, KC, (B) IL-17, IL-22 and IFNγ levels in culture supernatants of splenocytes from WT, NOD1$^{-/-}$ and NOD2$^{-/-}$ mice (n=5 mice per group) that were stimulated for either (A) 24 hours or (B) 5 days with heat inactivated *Aspergillus* conidia. (C) CFU remaining of *A. fumigatus* (2x10^6) following exposure for 24 hours to WT, NOD1$^{-/-}$ and NOD2$^{-/-}$ BMDMs (1x10^5) (D) ROS release by wild-type, NOD1$^{-/-}$ and NOD2$^{-/-}$ BMDMs following exposure to zymosan. Timepoints were compared for significance by two-way ANOVA. (E) area under the curve of the ROS luminescence data. Data in bar plots is represented as mean ± SEM and means were compared using the Mann Whitney U test. ud = undetectable.

Human NOD2 deficiency results in reduced innate and adaptive cytokine responses to *A. fumigatus*

Since we observed an interesting phenotype of protection with NOD receptor deficiency in mice, we decided to further investigate these receptors in human cells. We first investigated the host response against *Aspergillus* within a background of human NOD2 deficiency. The 1007 frame shift mutation is highly associated with Crohn's disease and results in a defective NOD2 receptor [21]. PBMCs from patients with Crohn's disease that were deficient for the NOD2 receptor due to homozygous carriage of a frameshift due to a insertion of cystein at position 1007 (1007finsC) were obtained, and were stimulated with different morphotypes of *A. fumigatus*. Compared to wild-type controls, NOD2 deficient PBMCs demonstrated lower IL-1β and TNFα responses to the different morphological forms of *A. fumigatus* (Figure 5A). Healthy volunteers demonstrated synergism upon co-stimulation with MDP whereas the NOD2 deficient patients demonstrated no change from the stimulation with *Aspergillus* alone, which conforms the complete NOD2 deficiency of these human cells (Figure 5B). Moreover, NOD2 deficient PBMCs showed a significant reduction in the ability to generate T-cell cytokines IL-17, IL-22 and IFNγ (Figure 5C). These reduced cytokine responses correlated with a reduced induction of IL-17$^+$, IL-22$^+$ and IFNγ$^+$ CD4 T-cells within the PBMC population of NOD2 deficient individuals (Figure 5D).

We next investigated polymorphisms in NOD2. Individuals with a heterozygous genotype for the 1007fins polymorphism (rs2066847) demonstrated significant lower IL-1β and a trend towards lower TNFα and IL-6 in response to live *Aspergillus* (Figure 6A). The decreased IL-1β induction in these individuals also correlated to significantly lower IL-17 responses in individuals carrying the

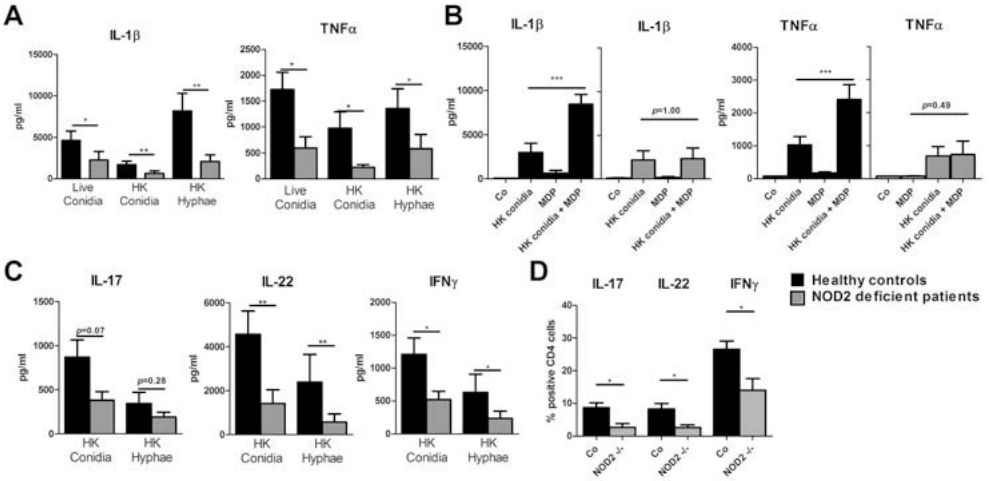

Figure 5 | Human NOD2 deficiency results in reduced innate and adaptive cytokine responses to *A. fumigatus*
(A) IL-1β and TNFα levels in culture supernatants of PBMCs from healthy controls (black bars) and NOD2 deficient patients (grey bars) that were either stimulated for 24 hours with *Aspergillus fumigatus* live conidia, HI conidia or HI hyphae for induction of IL-1β and TNFα (n=6 donors) or (B) stimulated with HI conidia, MDP or a combination of HI conidia and MDP (n=9 donors). (C) IL-17, IL-22 and IFNγ levels after 7 days of stimulation with HI conidia or HI hyphae (n=6 donors). (D) IL-17$^+$, IL-22$^+$ and IFNγ$^+$ CD4 T-cell populations (n=5 donors) after 7 days stimulation with HI conidia shown as percentage from total CD4 cells.. The bars represent Means ± SEM and the means of both groups were compared for significance using the Mann Withney U test *p*-values of statistical tests are shown within the graphs.

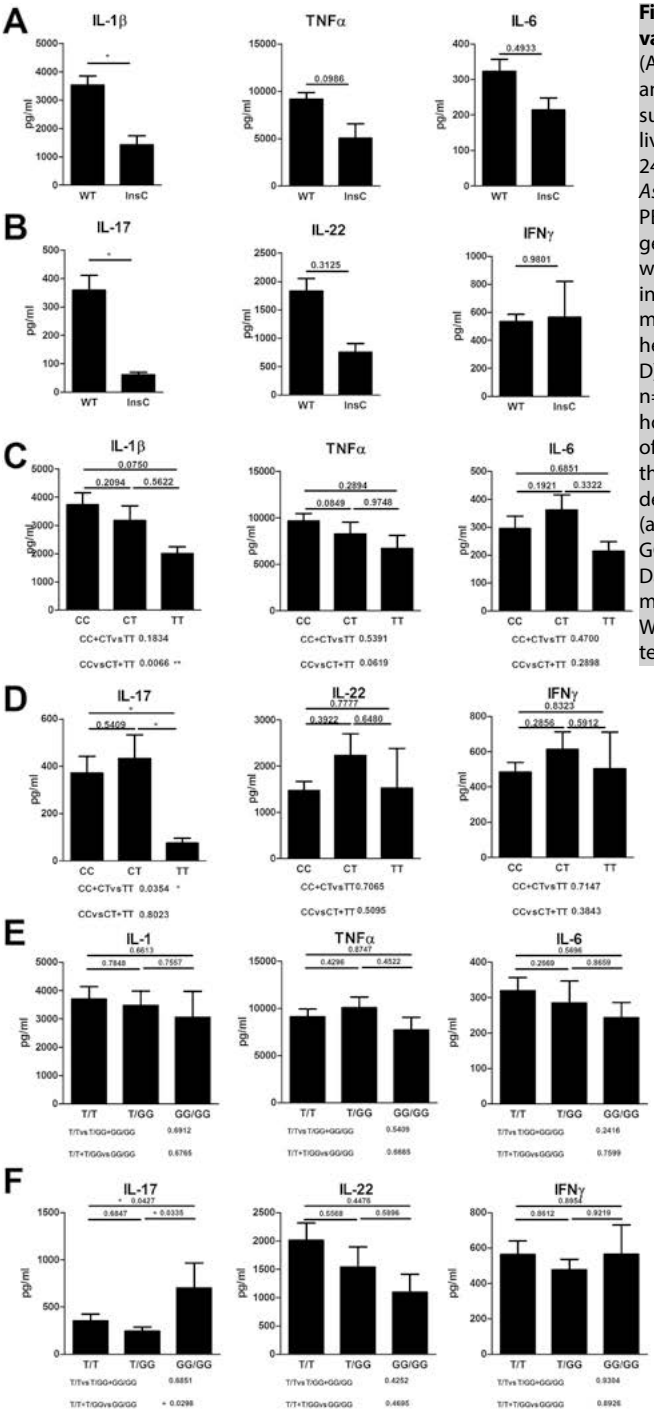

Figure 6 | NOD1 and NOD2 genetic variants

(A-F) IL-1β, TNFα, IL-6, IL-17, IL-22 and IFNγ levels measured in culture supernatants of PBMCs simulated with live (A, C and E) *Aspergillus* conidia for 24 hours or (B, D and F) heat killed *Aspergillus* conidia for 7 days. The PBMCs of individuals with various genotypes of the NOD2 receptor were compared. These genotypes included (A and B) the 1007finsC mutation (ancesteral;Wt n=62 and heterozygous;insC n=4) and (C and D) the P268S mutation (ancesteral;CC n=36, heterozygous;CT n=28 and homozygous;TT n=4). (E,F) The PBMCs of individuals with various genotypes in the NOD1 ND1+32656 T/GG insertion/deletion allele were compared (ancesteral;T/T n=36, heterozygous;T/GG n=23 and homozygous;GG/GG n=5) Data is represented as mean ±SEM and means were compared using the Mann Whitney U test, *p*-values of statistical tests are shown within the graphs.

C insertion on one allele (Figure 6B). Interestingly *Aspergillus*-induced IFNγ production was not affected by this genotype. In addition to the 1007fins polymorphism, we investigated other common genetic variants within the *NOD2* gene namely: P268S (rs2066842), G908R (rs2066845) and R702W (rs2066844) polymorphisms. Individuals carrying the T allele for the P268S polymorphism induced significantly lower IL-1β and a trend towards lower TNFα responses in response to *Aspergillus* stimulation (Figure 6C). Additionally, the TT genotype was associated with a significantly lower IL-17 production when comparing the response of these individuals with the GG and GT genotypes (Figure 6D). Individuals that were heterozygous for the G908R and R702W polymorphisms did not demonstrate altered cytokine responses to *Aspergillus* (data not shown). Homozygous G908R and R702W polymorphisms were not represented within our cohort.

The ND1+32656 T/GG mutation in NOD1 has been associated with inflammatory bowel disease [22] and asthma [23] previously. Since it involvement in inflammatory disease we investigate using this polymorphism whether this genetic variant could reveal a role for NOD1 in the human host response to *Aspergillus.* We stimulated PBMCs of individuals carrying the ND1+32656 GG insertion and compared the cytokine response to carriers of the ND1+32656 T deletion (wild-type). Individuals that were heterozygous or homozygous for the ND1+32656 GG insertion variant had IL-1β, TNFα and IL-6 responses compared to wildtype (Figure 6E). However, IL-17 was significantly higher in individuals having the GG/GG genotype, but IL-22 and IFNγ were not significantly affected by the presence of this polymorphism (Figure 6F).

Discussion

PRRs are key players in inducing antifungal host responses during invasive aspergillosis by inducing cytokine responses and facilitating phagocytosis. Extracellular PRRs, such as Toll-like receptors and C-type lectin receptors, have been extensively described in inducing these responses in response to *Aspergillus* [2]. However, there is only limited evidence for a role of the intracellular NACHT-LRR receptors (NLRs) in the host defence against *A. fumigatus* [6-8]. In the current report we investigated the role the NOD receptors in the host response against *A. fumigatus* and susceptibility to invasive aspergillosis.

Immunosuppression that is needed for the treatment of cancer and autoimmune disorders, makes patients highly susceptible to invasive fungal infections such as invasive aspergillosis. Cyclophosphamide is an immunosuppressive drug used to suppress the immune system in mice and render them highly susceptible to develop infections with *Aspergillus fumigatus*. This immunosuppression allows a low dose of intranasally administered *Aspergillus* conidia, which in immunocompetent mice would have been quickly cleared, to induce an invasive infection that is lethal in days [20]. In the current study we observed that NOD1 and NOD2 deficient mice were protected against aspergillosis while being immunosuppressed by cyclophosphamide. That a deficiency in pattern recognition receptors can protect against lethal aspergillosis is a striking observation. Especially when bearing in mind that it is challenging to protect immunosuppressed mice even with available potent antifungal therapies , which often requires combinational therapies to achieve survival of the mice [24,25]. Although NOD1 and NOD2 deficient mice are both protected against *Aspergillus* infection,

there is a big contrast between these mice. NOD1$^{-/-}$ mice quickly resolve the infection, whereas NOD2$^{-/-}$ mice developed severe symptoms and recovered significantly slower. This is in line with a previous study that demonstrated that *Staphylococcus aureus* pneumonia in NOD2 deficient mice was recovery than controlled the pneumonia then in wild-type animals [26]. However, such a protective effect during an infection has, to our knowledge, not been described for NOD1 deficiency. In contrast to NOD2 deficient mice, the NOD1 deficient mice demonstrated rapid fungal clearance following the infection, which was associated with no histological damage and fungal outgrowth in the lungs following lethal *Aspergillus* infection. Moreover, NOD1$^{-/-}$ cells demonstrated an increased capacity to produce pro-inflammatory cytokines. In line with these data a previous study demonstrated that NOD1 deficient mice are more prone to develop inflammation in a model for arthritis [27], and the ND1+32656 GG insertion polymorphism is associated with increased pro-inflammatory capacity of human cells and an increased clinical severity in patients with arthritis [28]. However, the NOD1 receptor was found to play an important role in host defence against bacterial pathogens such as *Pseusomonas aeruginosa* [29], *Shigella flexineri* [30] and *Helicobacter pylori* [31] where NOD1 was required for an effective cytokine response [29,30] and killing of the pathogen [29,31]. In contrast to these studies, our data shows that NOD1 deficiency results in an increased pro-inflammatory cytokine response and increased fungal killing, suggesting that NOD1 exerts an inhibitory effect on the antifungal host response against *Aspergillus*. How NOD1 deficiency enhances these mechanisms remains to be determined. We observed that NOD1 deficient macrophages demonstrate an enhanced oxidative burst upon stimulation with Zymosan. Although zymosan is recognised by dectin-1 and TLR2 [32], the NOD receptors are known to have interplay with TLRs via their downstream kinase RICK, and in particular with TLR2 [27,33-35]. Deficiency of NOD1 could therefore very well impact ROS release induced via dectin-1 and TLR2. ROS is crucial for the host defence against *Aspergillus*, which is illustrated by patients with chronic granulomatous disease with a defect in NADPH dependent ROS production are highly susceptible to infections with *Aspergillus*[36,37]. In addition, ROS has been shown to be involved in activation of the inflammasome by *Aspergillus* [38] which could explain the enhanced cytokine release by NOD1$^{-/-}$ cells. This potent protective effect of NOD1 deficiency makes it tempting to suggest blockade of NOD1 as a novel treatment strategy for invasive aspergillosis. Currently, no pharmacological inhibitors are available to block NOD1 in vivo, but recently small molecule inhibitors have been identified that could potentially be used for therapy [39].

We also found that NOD2 deficient mice were protected against invasive aspergillosis. However, these mice demonstrated a different phenotype than NOD1 deficient mice. We did not observe an enhanced oxidative burst as in the NOD1$^{-/-}$ cells. Moreover, cytokine responses in human and murine NOD2 deficient cells were decreased. This decreased inflammatory response could potentially explain why NOD2$^{-/-}$ mice are protected against IA. It has repeatedly been described that excessive inflammation and Th17-mediated inflammation can result in detrimental immunopathology during *Aspergillus* infections in mice [40-42]. NOD2 activation was previously demonstrated to play a significant role in activating the Th17 response [43]. In line with this we found that human NOD2 deficiency results in decreased *Aspergillus*-induced cytokine responses and T-cell proliferation, including IL-17^{+} cells. In addition, NOD2 deficient mice demonstrated less inflammation than wild-type mice. These

data suggest that, compared to wild-type, NOD2$^{-/-}$ mice are better able to cope with the *Aspergillus* infection due to their reduced inflammatory responses.

It is interesting that deficiency of NOD1 and NOD2 have different phenotypes in terms of disease, cytokine production, phagocytic capacity and ROS release, when considering that these receptors interact with each other through their shared downstream co-receptor RICK. A potential explanation for the differential phenotype is that both NOD1 and NOD2 compete for RICK as was previously proposed to explain differential regulation of inflammation by NOD1 and NOD2 in streptococcal cell wall fragments induced arthritis [27]. Another explanation for differential regulation by NOD1 and NOD2 is that one, or both, of these receptors can, in addition to RICK, bind an alternative signalling cascade. In line with this it has previously been demonstrated that NOD2 can additionally signal through the intracellular adaptor CARD9 [44], which has previously been associated with host response against fungi [45,46].

An important question that remains to be answered is how NOD1 and NOD2 are involved in the host defence against *Aspergillus*. It could be hypothesised that these receptors directly sense fungal PAMPs or that they modulate responses of other PRRs that are involved in recognition of *Aspergillus*. The NOD receptors have been discovered as receptors for derivates of bacterial peptidoglycan. NOD2 recognizes MDP which is present in the peptidoglycan of both gram positive and negative bacteria [9,11,12], and NOD1 recognizes diaminopimelate-containing GlcNAc-MurNAc-tripeptide muropeptide (GM-TriDAP) which is present in the peptidoglycan of gram negative bacteria [10,12,13]. However, fungi such as *Aspergillus fumigatus* have neither of these peptidoglycan derived molecules, but their cell wall is rich in PAMPs [47] that might serve as potential ligands for NOD receptors. Although it has previously been shown that other fungi such as *Candida albicans* are not recognised by NOD2 [48,49], the cell wall structure chitin that is present in both *Aspergillus* and *Candida* is being suggested to target NACHT-LRR receptors (NLRs) [5,50]. Chitosan, a deacitylated form of chitin was found to activate the NLR, NOD-like receptor family, pyrin domain containing 3 (NLRP3) and thereby activate the inflammasome and induce IL-1β production, whereas chitin did not activate NLRP3 [50]. However, chitin was also found to induce IL-10 via TLR9, mannose receptor and NOD2 dependent mechanisms [5]. Suggesting that NLRs, such as NLRP3 and NOD2 play a role in recognition of fungal molecules such as chitin. However, further studies are required to further identify the PAMPs in *Aspergillus* that are recognised by NOD1 and NOD2. It is also possible that the NOD receptors are not directly involved in recognising *Aspergillus*, but rather coordinate the responses that are induced by other (membrane bound) PRRs. Both NOD1 and NOD2 have extensively described to synergise with TLRs [51-55]. By selectively modulating signals from PRRs that recognise *Aspergillus* it is possible that the NOD receptors differentially regulate the host response against *A. fumigatus*.

Collectively, we conclude that the absence of NOD1 enhances the protective host response against *Aspergillus* in an immunocompromised setting, by enhancing oxidative burst and fungal killing. In contrast NOD2 seems to be involved in mounting innate and adaptive cytokine responses against *Aspergillus.* This paves the way for the development of new treatment strategies for invasive aspergillosis that target NOD1.

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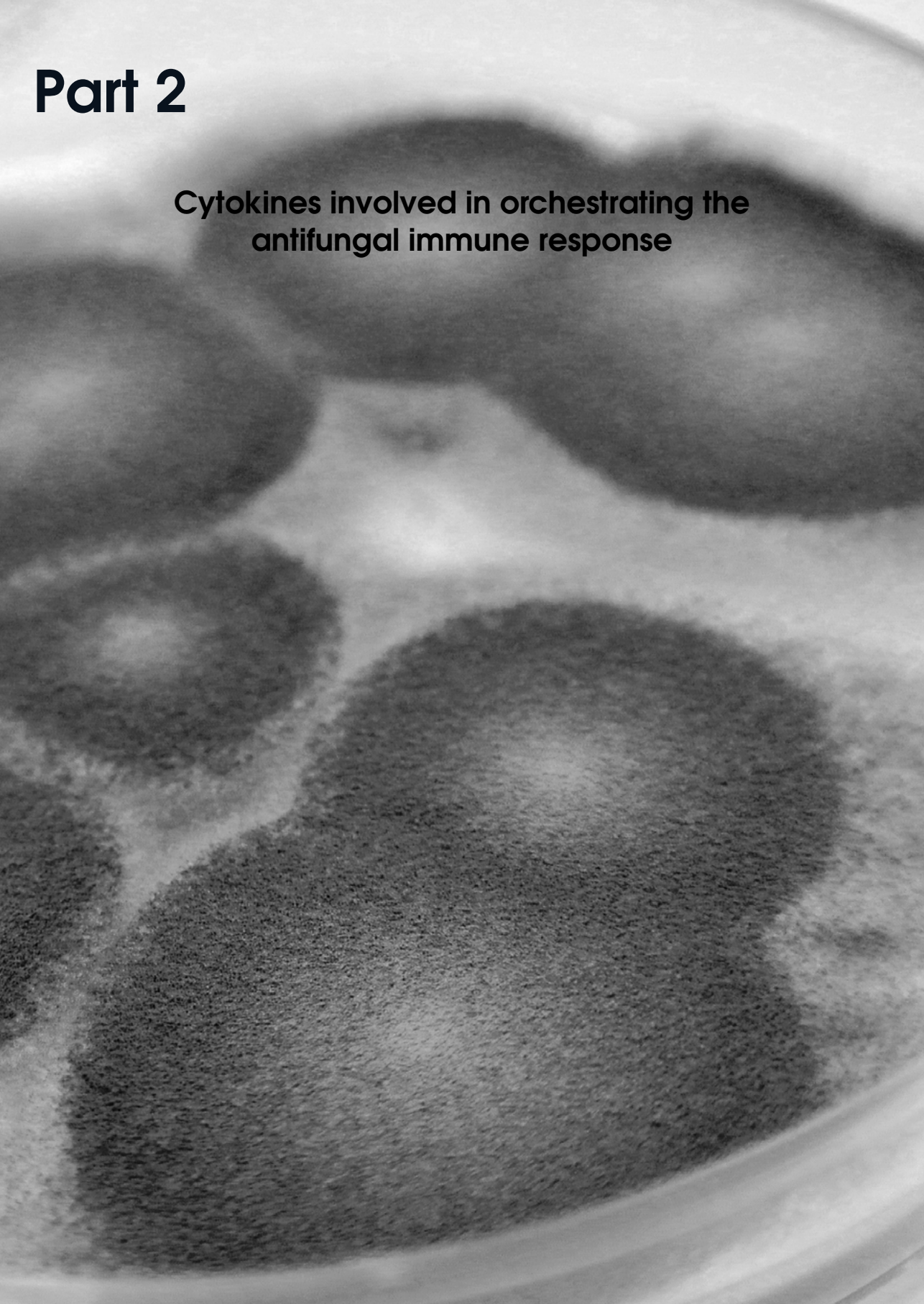
Part 2

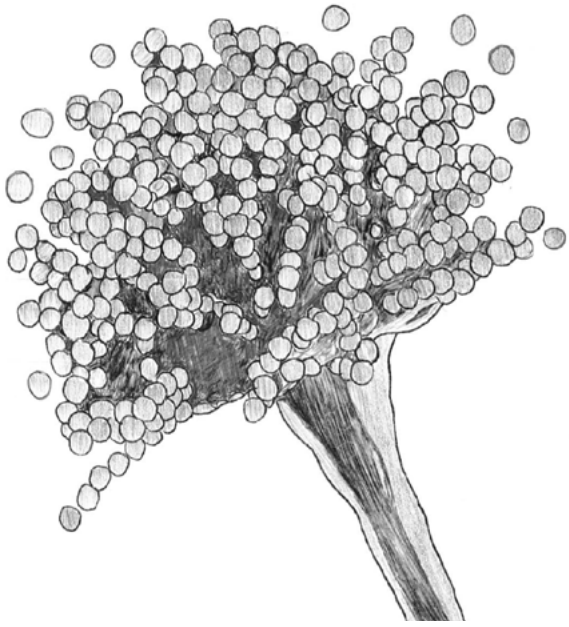
Cytokines involved in orchestrating the antifungal immune response



Chapter 7

The role of interleukin-1 family members in the host defence against *Aspergillus fumigatus*

Mark S. Gresnigt and Frank L. van de Veerdonk



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Abstract

The IL-1 family consists of 11 members, which all play significant roles in regulating inflammatory responses in the host. IL-1α and IL-1β exert potent pro-inflammatory effects and are key players in the recruitment of neutrophils to the site of inflammation. Protective anti-*Aspergillus* host responses during the early stages of invasive aspergillosis are critically dependent on neutrophil recruitment, and several lines of evidence support that there is an important role for IL-1 in this process. However, IL-1-mediated inflammation needs to be tightly regulated, since uncontrolled inflammation can result in inflammatory pathology and thereby be detrimental for the host. *Aspergillus*-induced IL-1-mediated inflammation could therefore be amendable for IL-1 blockade under specific circumstances. This review describes the current understanding of the role of IL-1 family members in the host response against *Aspergillus fumigatus* and highlights the importance of balanced IL-1 responses in aspergillosis.

Introduction

Since its discovery, interleukin (IL)-1 has been found to be a crucial cytokine in the host defence against a broad range of pathogens, ranging from bacteria to parasites and fungi. Initially, IL-1 was described as a single cytokine, but to date the IL-1 cytokine family consists of 11 members [1]. In this family, the classical IL-1 cytokines are IL-1α and IL-1β, which are often described together as IL-1. IL-1β is primarily produced by innate immune cells such as monocytes, macrophages and dendritic cells upon activation. IL-1α also is induced by cells of the innate immune system, but also more widely expressed in different cell types, including pulmonary epithelial cells where it is released upon cell death [2]. Over the last years, the cytokines from the IL-1 family, and in particular IL-1β, have been studied for their role in antifungal host response against *Aspergillus fumigatus.* This review summarizes the findings reported on the role of the IL-1 family members in the antifungal host response against *A. fumigatus.*

IL-1 expression, processing and secretion induced by *A. fumigatus*

IL-1β is a potent pro-inflammatory cytokine; therefore, its release by immune cells is tightly regulated. Transcriptional analysis of monocytes exposed to *A. fumigatus* demonstrated that resting conidia could induce *IL1A* and *IL1B* expression; however, swollen conidia, germinated conidia and hyphae were more potent in inducing *IL1A* and *IL1B* expression [3]. Additional studies have shown that hyphal fragments [4] or live conidia [5] but not (inactive) resting conidia induce *IL1B* transcription. These data suggest that pathogen associated molecular patterns (PAMPs) that are being exposed on the *Aspergillus* cell wall following germination are different and are more potent in inducing IL-1.

An important aspect of IL-1β is that its mRNA translates into an inactive precursor, namely pro-IL-1β, which requires processing to become fully bioactive IL-1β. This processing can be performed by a protein complex called the inflammasome that induces activation of caspase-1, which proteolytically removes the pro- part [6]. *Aspergillus* induces inflammasome activation dependent on reactive oxygen species (ROS) and potassium influx [4]. PAMPs in the *Aspergillus* cell wall are recognized by pattern recognition receptors (PRRs), such as C-type lectin receptors (CLRs) or Toll-like receptors (TLRs) [7]. Both MyD88 (downstream adaptor of TLRs and the IL-1 receptor) and spleen tyrosine kinase (Syk) (downstream adaptor molecule of CLRs, like dectin-1) were found to play a significant role in pro-IL-1β induction and secretion of mature IL-1β in response to *Aspergillus* [4]. However, neutralization of Syk, but not MyD88, was found to prevent inflammasome activation [4]. This suggests that MyD88 only regulates *IL1B* transcription, while Syk regulates both its transcription and processing. Interestingly, when pro-IL-1β is released extracellularly, it can also be processed by inflammasome-independent mechanisms, such as cleavage by neutrophil serine proteases like proteinase-3 [8]. Several reports have demonstrated that the dectin-1/Syk [9,10] and dectin-2-Syk [5] pathways play a prominent role in the induction of IL-1 responses by *A. fumigatus*. Blockade of dectin-1 decreases *Aspergillus*-induced IL-1α and IL-1β [9] and dectin-1$^{-/-}$ mice fail to induce strong IL-1α and IL-1β responses [10]. Furthermore, dectin-2 regulates the induction of *IL1B* expression as well as IL-1β protein secretion [5]. Macrophages isolated from TLR4- and TLR2-deficient mice have impaired

IL-1α and IL-1β production in response to *A. fumigatus* conidia [11]. Interestingly, after interaction with germinated *Aspergillus* conidia, TLR4-/-macrophages showed no impaired IL-1 response, suggesting that the ligand for TLR4 on the hyphal cell wall that mediates induction of the IL-1 response is either absent or masked. Figure 1 visualizes the mechanism of IL-1 induction, processing and release of innate immune cells in response to *A. fumigatus*.

Effects of IL-1 signalling in host defence against *Aspergillus*

After its release, IL-1 can bind to the IL-1 type1 receptor (IL-1R1), which is followed by the recruitment of IL-1 receptor accessory protein (IL-1RAcP) [12]. Recruitment of IL-1RAcP to IL-1R1 results in activation of signal transduction pathways, leading to activation of MyD88, thereby exerting potent inflammatory activities. The recruitment of inflammatory cells, particularly neutrophils, is driven by IL-1 signalling [12,13]. During *Aspergillus* infection, IL-1β can be detected in the lung [14], where it can directly induce chemokine release by pulmonary epithelial cells, which results in neutrophil recruitment [13]. In addition to the induction of innate immune responses, IL-1 plays a key role in induction of T-helper cell responses by *A. fumigatus* [15,16]. The *Aspergillus*-induced T-helper cytokines IL-17 and IL-22 are tightly regulated by IL-1, since IL-1R neutralization by recombinant IL-1Ra completely abolishes these responses [15]. These responses amplify the pro-inflammatory effects of IL-1, as IL-17 plays a crucial role in neutrophil recruitment, and IL-22 regulates production of antimicrobial peptides by pulmonary epithelial cells [17,18]. Additionally, IL-1R blockade demonstrated that IL-1 also regulates *Aspergillus*-induced Th1 (IFNγ) [16,19] and Th2 (IL-5 and IL-13) responses in humans [20].

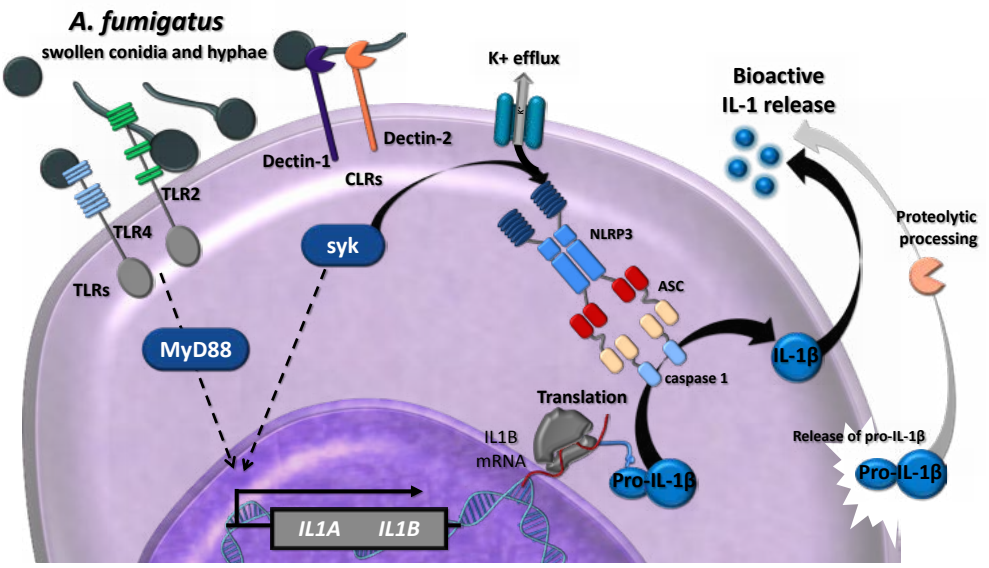

Figure 1 | IL-1 processing and secretion induced by *A. fumigatus*
Schematic overview of *IL1* transcription induction by *Aspergillus fumigatus* through MyD88 and Syk-dependent pathways. Subsequently IL1B mRNA is translated into the inactive precursor pro-IL-1β which can subsequently be processed into its active form by the protein complex the inflammasome. Upon cell damage, pro-IL-1β can be released which can be processed extracellular by serine proteases such as proteinase-3.

Pro-inflammatory effects of IL-1 in aspergillosis

Monocyte-derived macrophages from patients with chronic cavitary pulmonary aspergillosis (CCPA) persistently express *IL1A* and *IL1B* upon stimulation with *Aspergillus,* compared to healthy controls who did not demonstrate this phenomenon [21]. This suggests that constant IL-1 expression could play an important role in the pathogenesis of CCPA due to continuous activation of the pro-inflammatory immune response.

Although IL-1 signalling is generally associated with protection against infection [22], a previous report showed that IL-1R1$^{-/-}$ mice have no attenuated survival upon challenge with *A. fumigatus*; these mice demonstrated even a slightly increased survival and a significantly reduced fungal burden [23]. In contrast to this observation, IL-1R1$^{-/-}$ mice have recently been described to be highly susceptible to *Aspergillus* infection, showing increased fungal growth, dissemination and pulmonary pathology [24]. These conflicting observations could be explained by the different aspergillosis models used in these studies. Since IL-1 can promote immunopathology in the lung, it would be beneficial to have no IL-1 signalling in a model where the *Aspergillus* infection induces inflammatory pathology. However, when the model is highly dependent on IL-1-induced neutrophil recruitment, it will be detrimental for the host to have no IL-1 signalling, which is required to promote early fungal clearance (figure 2). This differential role of the IL-1 axis is particularly illustrated within dectin-1$^{-/-}$ mice. As previously mentioned, dectin-1$^{-/-}$ mice fail to induce strong IL-1α and IL-1β responses, which results in a failure to induce an adequate IL-17 response and increased mortality in a model of invasive aspergillosis (IA) [10]. However, in an allergic model , with chronic exposure to *A. fumigatus,* the dectin-1$^{-/-}$ mice have decreased IL-1β levels, which is associated with reduced IL-22-associated immunopathology [25].

Anti-inflammatory effects of IL-1Ra

IL-1 receptor antagonist (IL-1Ra) is a potent endogenous anti-inflammatory cytokine that regulates IL-1 bioactivity by competing with pro-inflammatory IL-1 molecules (IL-1α and IL-1β). Upon binding of IL-1Ra to the IL-1R1, there will be no recruitment of IL-1RAcP and no IL-1 signal transduction [12,26]. The *A. fumigatus* cell wall polysaccharide galactosaminogalactan (GAG) exerts potent anti-inflammatory effects by diminishing neutrophil recruitment and favouring fungal infection [27]. Recent studies exploring these anti-inflammatory effects have shown that GAG is a crucial adherence factor [28] and could specifically induce IL-1Ra, thereby inhibiting the IL-1 axis and induction of T-helper responses, resulting in an increased susceptibility against aspergillosis [16]. This supports the concept that in early stages of *Aspergillus* infection deficient IL-1 signalling can be detrimental, because in these stages it is crucial to control fungal growth by neutrophils, which is dependent on IL-1.

A group of patients that are specifically prone to develop severe infections with *Aspergillus* are patients with chronic granulomatous disease (CGD) [29,30]. These patients have a defect in NADPH oxidase-dependent ROS production and fail to induce a form of non-canonical autophagy, namely LC3-associated phagocytosis (LAP) [31,32]. LAP is an important mechanism that is required for effective microbial killing and antigen presentation [33,34]. Despite the fact that these patients are unable to induce NADPH-dependent ROS, these patients demonstrate increased inflammasome activation,

which correlates with increased IL-1β production [35]. The blockade of IL-1 signalling by IL-1Ra in these patients resulted in reduced inflammation [36]. This is in line with the concept that uncontrolled increased IL-1-mediated inflammation is detrimental for the host and can be a target in CGD.

Monocytes from CGD patients that are stimulated in the presence of IL-1Ra demonstrate a restored *Aspergillus*-induced LAP. Moreover, IL-1Ra administration reduces fungal burden and rescues p47$^{phox-/-}$ (CGD) mice in a model of IA [32]. Thus, IL-1Ra could contribute to increased fungal clearance in the setting of a defect in LC3-associated phagocytosis, such as in patients with CGD, by inducing autophagy [32]. The mechanism by which IL-1Ra induces non-canonical autophagy remains to be identified.

IL-1 polymorphisms as genetic susceptibility markers for aspergillosis

Polymorphisms within genes of the immune system that are common in the general population can influence the ability to mount an effective antifungal host response. Analysis of polymorphisms within the IL-1 gene cluster consisting of *IL1A, IL1B* and *IL1RN* revealed that the *IL1B* -511T allele (rs1143627) correlates with incidence of IA in haematological patients [37]. Although, *IL1A* and *IL1RN* polymorphisms were not directly associated with IA, a haplotype consisting of the *IL1RN* VNTR2 (rs380092), *IL1A* -889C (rs1800587) and *IL1B* -511T alleles was highly associated with the development of IA. In CCPA, an intronic insertion deletion in *IL1B* (rs3917354) and a 3'UTR polymorphism in *IL1RN* (rs4252041) were found to be associated with the development of CCPA when comparing CCPA patients with healthy controls [21].

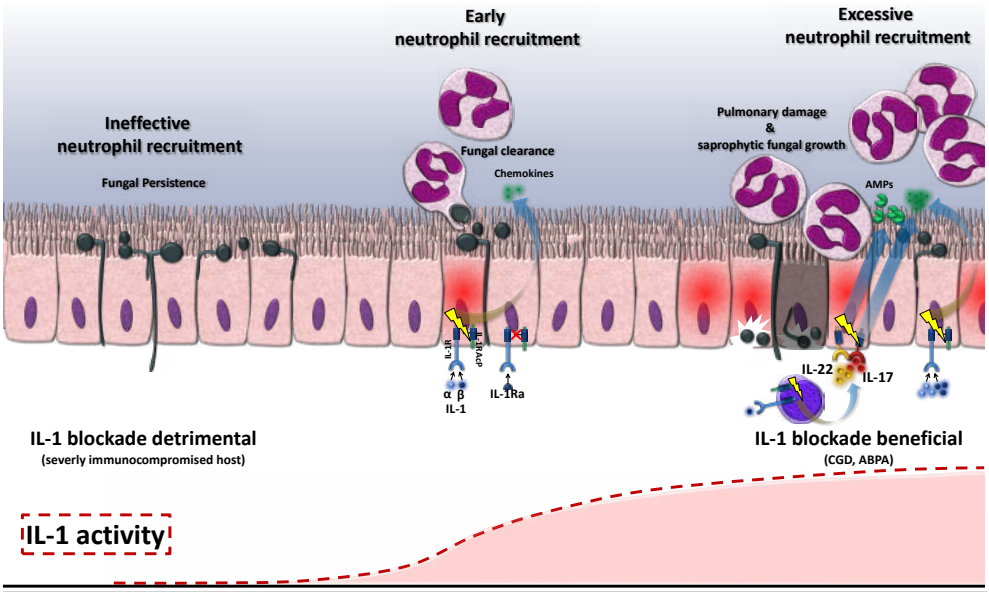

Figure 2 | Beneficial and detrimental effects of IL-1 depending on the type and stage of *Aspergillus* disease
When the host response is dependent on neutrophils to clear *Aspergillus* infection, such as during the early stages of invasive pulmonary aspergillosis, IL-1 is crucial for an optimal protective host defence. However, in the setting of an exaggerated inflammatory response mediated by *Aspergillus,* IL-1 can play a detrimental role by maintaining neutrophil influx, increased Th17 responses leading to tissue damage.

Novel IL-1 family members

The IL-1 family not only consists of IL-1α, IL-1β and IL-1Ra but also other IL-1 family cytokines, such as IL-33 and IL-18. A subset of the new IL-1 family members has recently been renamed according to new insights in their biological function, namely IL-36, IL-37 and IL-38 [38]. IL-36 consists of three agonistic cytokines, namely IL-36α (IL-1F6), IL-36β (IL-1F8) and IL-36γ (IL-1F9) and one antagonistic cytokine IL-36Ra (IL-1F5)[38]. IL-1 and IL-36 are quite similar in their biological function. IL-36 cytokines are agonists of the IL-1 receptor related protein 2 (IL-1Rrp2), now also known as the IL-36R, that requires, like the IL-1R1, IL-1RAcP as a co-receptor for signal transduction [39]. Furthermore, IL-36Ra inhibits signalling by binding to IL-36R competitively with the IL-36R agonists and preventing IL-1RAcP recruitment , which is similar to how IL-1Ra inhibits IL-1R1 signalling [40].

The IL-36 receptor signalling pathway plays a role in the host response against *A. fumigatus* by regulating the induction of T-cell cytokines such as IL-17 and IFNγ, but not the Th2 response [41]. IL-38 (IL-1F10) was shown to exhibit similar receptor-antagonizing functions as IL-36Ra in the induction of T-cell cytokine responses by *Candida albicans* [42]. However, it remains to be elucidated whether IL-38 also plays a role in the host response against *A. fumigatus*. IL-37 (IL-1F7) is a novel anti-inflammatory cytokine the IL-1 family. It binds the IL-18 receptor, but does not compete with IL-18 [43], and intracellularly it can form a functional complex together with Smad3 to suppress innate immune responses [44]. The role of IL-37 in antifungal host defence against *Aspergillus* is currently under investigation.

Future perspectives

The present data demonstrate the importance of IL-1 in controlling fungal clearance in *Aspergillus* infections, as well as a role for IL-1 in the inflammatory pathology of *Aspergillus*-associated diseases. Therefore, blocking IL-1 with the available biological anakinra, which is recombinant IL-1Ra, can be explored in *Aspergillus*-related diseases, such as IA in CGD or fungal asthma, but the timing and at what stage anakinra treatment should be given will be crucial. Predicting which patients are at high risk of developing IA could provide a powerful tool to stratify patients in need for intensive monitoring or prophylactic antifungal therapy. Based on the current literature and ongoing studies that demonstrate an association between IL-1 polymorphisms and susceptibility to aspergillosis, we suggest that IL-1 is a suitable candidate for predicting genetic susceptibility to aspergillosis.

The novel IL-1 family cytokines IL-36 and IL-38 could play a significant role in the host defence against *A. fumigatus*. These cytokines are differentially expressed within tissues and cell types, and therefore, they could be of specific interest in targeting them during inflammation. Although IL-36 cytokines were primarily found to play a role in the pathogenesis of psoriasis [45], they were also found to play a prominent role in inflammation and neutrophil influx in the lungs independent of IL-1α and IL-1β [46,47]. Furthermore, they play a role in allergen-induced inflammation in the lung [48] and were differentially regulated in patients with chronic obstructive pulmonary disease (COPD) exacerbations [49]. With this knowledge in hindsight, we suggest that these cytokines could potentially play an important role in fungal sensitization and allergic bronchopulmonary aspergillosis (ABPA), and might be promising targets in *Aspergillus*-related allergic diseases.

Acknowledgements

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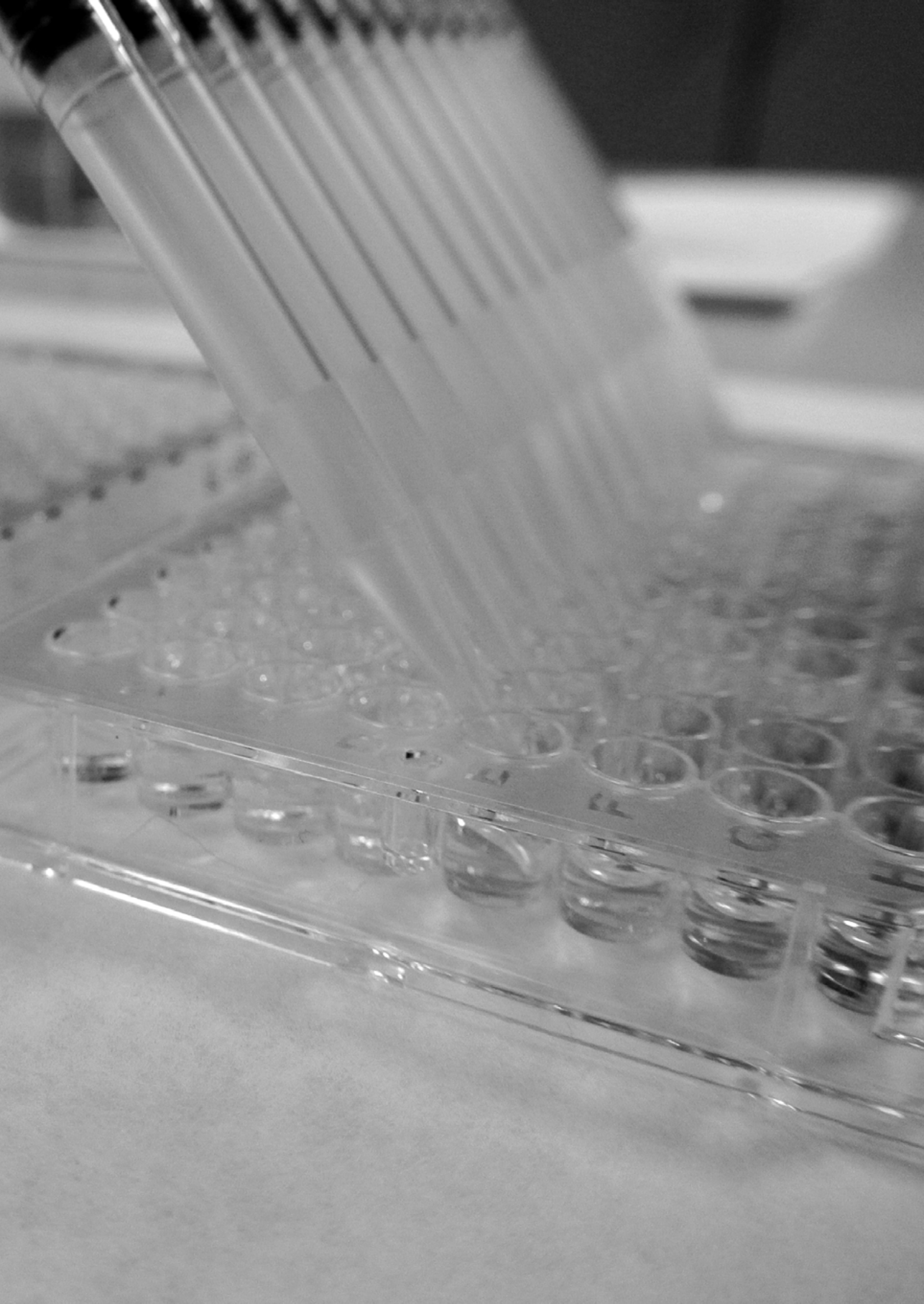

Chapter 8

A polysaccharide virulence factor from *Aspergillus fumigatus* elicits anti-inflammatory effects through induction of interleukin-1 receptor antagonist

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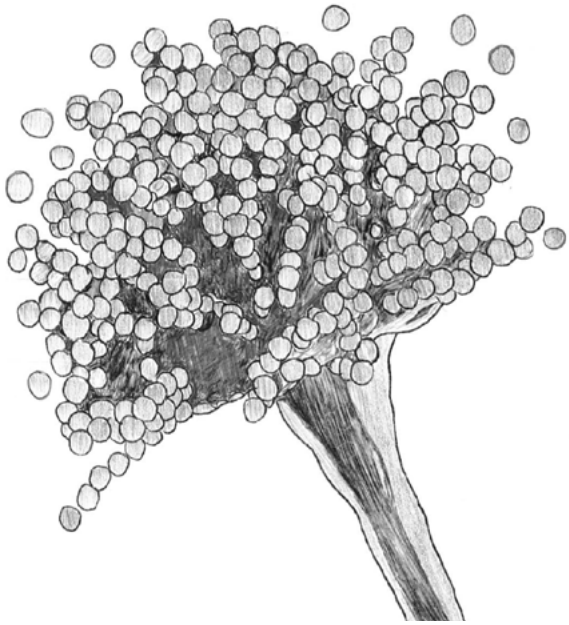

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Abstract

The galactosaminogalactan (GAG) is a cell wall component of *Aspergillus fumigatus* that has potent anti-inflammatory effects in mice. However, the mechanisms responsible for the anti-inflammatory property of GAG remain to be elucidated. In the present study we used *in vitro* PBMC stimulation assays to demonstrate, that GAG inhibits pro-inflammatory T-helper (Th)1 and Th17 cytokine production in human PBMCs by inducing Interleukin-1 receptor antagonist (IL-1Ra), a potent anti-inflammatory cytokine that blocks IL-1 signalling. GAG cannot suppress human T-helper cytokine production in the presence of neutralizing antibodies against IL-1Ra. In a mouse model of invasive aspergillosis, GAG induces IL-1Ra *in vivo*, and the increased susceptibility to invasive aspergillosis in the presence of GAG in wild type mice is not observed in mice deficient for IL-1Ra. Additionally, we demonstrate that the capacity of GAG to induce IL-1Ra could also be used for treatment of inflammatory diseases, as GAG was able to reduce severity of an experimental model of allergic aspergillosis, and in a murine DSS-induced colitis model. In the setting of invasive aspergillosis GAG has a significant immunomodulatory function by inducing IL-1Ra and notably IL-1Ra knockout mice are completely protected to invasive pulmonary aspergillosis. This opens new treatment strategies that target IL-1Ra in the setting of acute invasive fungal infection. However, the observation that GAG can also protect mice from allergy and colitis makes GAG or a derivative structure of GAG a potential treatment compound for IL-1 driven inflammatory diseases.

Author summary

Aspergillus fumigatus is an opportunistic pathogenic fungus that primarily causes infections in the immunocompromised host. It is known that *Aspergillus* employs various strategies to evade immune recognition by the hosts immune system. Recently, galactosaminogalactan (GAG), a new component of the *Aspergillus* cell wall, was discovered to have potent anti-inflammatory effects in mice making them more susceptible to *Aspergillosis*. In the current study we found that this anti-inflammatory property of GAG was due to its capacity to induce the potent anti-inflammatory cytokine interleukin-1 Receptor antagonist. This cytokine interferes with IL-1 signalling and thereby can reduce IL-1–induced immune responses such as T-cell responses. We also found that the induction of this anti-inflammatory cytokine by GAG correlates with increased fungal burden, and mice deficient for this cytokine were protected against aspergillosis. Additionally, we show that the capacity of GAG to induce the natural regulator of IL-1 signalling could be used in the treatment of IL-1–mediated disease such as allergy and colitis. Our study provides new insights on the immunoregulatory activity of GAG and opens up possibilities to exploit the anti-inflammatory potential of GAG as a therapy for inflammatory diseases.

Introduction

Aspergillus fumigatus is an opportunistic fungus that causes infections under specific conditions, of which secondary immunodeficiency is by far the largest risk factor for the development of invasive infections [1]. In order to initiate an effective host response against *Aspergillus*, recognition of conserved pathogen associated molecular patterns (PAMPs) by specific pattern recognition receptors (PRRs) is required.

A. fumigatus has a complex cell wall consisting of polysaccharides that play essential biological functions in fungal cell biology and host-pathogen interactions. Some of these polysaccharides are recognized by various PRRs expressed on human immune cells [2]. However, *A. fumigatus* employs various strategies to evade immune recognition. *Aspergillus* expresses surface molecules that shield PAMPs or can modulate TLR responses [3]. Several surface molecules and PAMPs of *A. fumigatus* have been characterized as being capable of modulating or suppressing the immune response. Rodlets and melanin, that are present on the conidial surface, shield PAMPs that elicit pro-inflammatory host responses [4,5]. In addition, β-glucan, α-glucan and galactomannan (GM) have been shown to modulate the host immune response [6].

Another cell wall component of *A. fumigatus* that is capable of modulating the immune response is galactosaminogalactan (GAG) [7]. GAG is not expressed on *Aspergillus* conidia, but is exposed when conidia start to germinate and was found to be present in the extracellular matrix that surrounds *Aspergillus* hyphae in aspergilloma isolated from patients and in experimental murine invasive aspergillosis [8]. Furthermore, GAG has been shown to serve as an adhesin of *Aspergillus* [9,10] and to shield β-glucan moieties on the cell wall [9]. This polysaccharide that is shed into the host environment during *Aspergillus* vegetative growth induces immunosuppressive effects that results in diminished neutrophil recruitment, which predisposes mice to *A. fumigatus* infection [7]. However, the mechanism through which GAG induces immunosuppressive effects as well as its capacity to induce similar immunosuppressive effects on the human immune response were unknown. Therefore, we investigated whether GAG can be immunosuppressive in the human host response against *A. fumigatus,* and we have systematically addressed the possible mechanisms responsible for the anti-inflammatory property of GAG.

Experimental procedures

Ethics Statement | All studies with human blood samples were conducted in the Radboud University Nijmegen Medical Centre and the use of healthy volunteers was approved by the institutional ethics review board. Peripheral venous blood samples from healthy volunteers were obtained after written informed consent was provided. All animal studies were conducted within the University of Perugia and were performed according to the Italian Approved Animal Welfare Assurance A–3143–01. Legislative decree 245/2011-B regarding the animal license was obtained by the Italian Ministry of Health lasting for three years (2011–2014). Infections were performed under avertin anesthesia and all efforts were made to minimize suffering.

Galactosaminogalactan | Galactosaminogalactan (GAG) was isolated from *A. fumigatus* culture supernatant and purified from the urea-soluble fraction as previously described [7]. Lyophilized GAG was resuspended in 10 mM HCl at 2 mg/mL and used in a final concentration of 10 µg/mL. Before using GAG in stimulation experiments it was incubated with polymixin B to neutralize potential contamination of lipopolysaccharide.

Stimuli and reagents | A clinical isolate of *Aspergillus fumigatus* V05-27, which has been previously characterized was used for stimulations [11]. Conidia and hyphae were prepared and heat-killed as previously described [6]. A concentration of $1x10^7$/mL was used in the experiments, unless otherwise indicated. Recombinant human IL-1β, IL-23, IL-12 and IL-18 were purchased from R&D Systems (Minneapolis, MN, USA) and were used in end concentrations of 100 ng/mL, 50 ng/mL, 10 ng/mL and 50 ng/mL respectively. Recombinant human (rh) IL-1Ra (Amgen, Inc., Thousand Oaks, CA, USA) was used to antagonize IL-1β signalling at a final concentration of 10 ng/mL. Anti-human IL-1Ra (R&D) was used to block IL-1Ra in a final concentration of 10 µg/mL, and was compared to isotype control.

PBMC isolation and stimulation | PBMCs were isolated as described previously [12]. The cells were counted using a particle counter (Beckmann Coulter, Woerden, The Netherlands) and the cell number was adjusted to $5x10^6$/mL. PBMCs were plated in 96-well round-bottom plates (Corning, NY, USA) at a final concentration of $2,5x10^6$/mL and in a total volume of 200 µL. Cells were pre-stimulated for 1 hour with medium or 10 µg/mL GAG. Following prestimulation, the PBMCs were stimulated with culture medium, heat killed *A. fumigatus* conidia ($1x10^7$/mL), IL-1β/IL-23 (100 and 50 ng/mL respectively) or IL-12/IL-18 (10 and 50 ng/mL respectively). These experiments were also performed in the presence of 10 µg/mL anti-IL-1Ra antibody or isotype control. Plates were incubated at 37°C, 5% CO_2 for 24 hours, 48 hours or 7 days. 7 day cultures were supplemented with 10% human serum. In this serum we detected anti-GAG antibodies as described previously [7]. After incubation, culture supernatants were collected and stored at -20°C until cytokine measurements were performed.

IL-1 bioassay | The murine cell line NOB-1 responds to both human or mouse IL-1 by production of IL-2, furthermore these cells are unresponsive to other cytokines like tumour necrosis factor (TNF), colony stimulating factors (CSFS), IL-3, IL-5, IL-6 and IFNγ [13]. NOB-1 cells were plated in 96-well flat-bottom plates at a final density of $1x10^6$ cells/mL and were stimulated for 24 hour using culture supernatants of unstimulated PBMCs or PBMCs stimulated in presence of GAG (GAG conditioned medium). After 24 hours of incubation at 37°C, 5% CO_2 the culture supernatants of the NOB-1 cells were collected and IL-2 production by the NOB-1 cells was measured by ELISA (R&D systems).

Cytokine measurement | Cytokines were measured using commercially available ELISAs (R&D Systems)(Biolegend, San Diego, CA, USA) (Sanquin, Amsterdam, The Netherlands) according to the protocols supplied by the manufacturer. IL-1α, IL-1β, TNFα, IL-6, IL-8, IL-1Ra and IL-10 were measured after 24 hours, and IL-5, IL-9, IL-13, IL-17, IL-22 and IFNγ were measured after 7 day stimulation.

Mice | Female, 8- to 10-weeks old, BALB/c (wild-type, WT) mice were purchased from Charles River (Calco, Italy). Breeding pairs of homozygous *IL1RN*$^{-/-}$ mice on the BALB/c background, were kept under specific-pathogen free conditions at the breeding facilities of the University of Perugia, Perugia, Italy. Experiments were performed according to the Italian Approved Animal Welfare Assurance 229-2011-B.

Fungal infection, allergy and treatment | Viable conidia from the *A. fumigatus* Af293 strain were obtained as described [14]. For infection mice were anesthetized in a small plastic cage, containing 3% Isofluoran (Isofluran Forene Abbot Scandinavia AB, Solna) before intranasal (i.n.) instillation of a suspension of 2x10^7 resting conidia/20 µL saline. Mice were treated with 250 µg/kg i.n. of GAG the day of infection and on days 1 to 3 post infection. Mice were monitored for survival, fungal growth (colony forming unit/organ, mean ± SEM), as described [15], histopathology, myeloperoxidase (*Mpo*) and *IL1RN* mRNA expression in lung cells and IL-1Ra production. For histology, sections (3–4 µm) of paraffin-embedded tissues were stained with periodic acid-Schiff (PAS) reagent. For allergy, mice received an i.p. and s.c. injection of 100 µg of *A. fumigatus* culture filtrate extract (CCFA) dissolved in incomplete Freund's adjuvant (Sigma) followed by two consecutive intranasal injections (a week apart) of 20 µg CCFA. A week after the last intranasal challenge, mice received 10^7 *Aspergillus* resting conidia and evaluated a week later (16424201). GAG (250 µg/kg i.n.) or vehicle alone was administered daily, for a week, in concomitance with the *Aspergillus* infection.

Collection of broncho-alveolar lavage (BAL) fluid | Lungs were filled thoroughly with 1 mL aliquots of pyrogen-free saline through a 22-gauge bead-tipped feeding needle introduced into the trachea. The lavage fluid was collected in a plastic tube on ice and centrifuged at 400 g, 4°C, for 5 minutes. For differential BAL cell counts, cytospin preparations were made and stained with May-Grünwald Giemsa reagents (Sigma-Aldrich). At least 200 cells per cytospin preparation were counted and the absolute number of each cell type was calculated. Photographs were observed using a BX51 microscope (Olympus, Milan, Italy) and images were captured using a high-resolution DP71 camera (Olympus).

Dextran sulfate sodium–induced colitis | Mice received either regular drinking water (control) or 2.5% dextran sulfate sodium (DSS) in drinking water for 7 days and then allowed to recover by drinking water alone for an additional 7 days. GAG was given intraperitoneally (1 mg/kg) daily for a week. Weight changes were recorded daily, and the day after the 7-days of rest mice were killed and tissues were collected for histology and cytokine analysis. Colonic sections were stained with H&E [16]. To assess colitis severity, stool and histological scores were used that recently were introduced and proven sensitive to experimental therapy [17].

Cell purification and cell cultures | Purified peritoneal CD11b$^+$ Gr-1$^+$ polymorphonuclear neutrophils (PMNs) (>98% pure on FACS analysis) were obtained as described [18]. Lung epithelial

cells were isolated as described [19]. Murine macrophages were isolated from total lung cells after 2 hours plastic adherence at 37°C. PMNs, epithelial cells and macrophages were exposed to non-opsonized *Aspergillus* conidia at the ratio of 1:1 or LPS (10 ng/mL) at 37°C for 1 hour in the presence of different concentrations (1 or 20 µg/mL) of GAG for 18 hours before the assessment of *IL1RN* mRNA expression.

Statistical analysis | The differences between the various stimulations were analysed with the Wilcoxon signed rank test (*p*-value of <0.05 was considered statistically significant). All experiments were performed at least twice and data represent cumulative results of all experiments performed and are presented as mean ± standard error of the mean (SEM) unless otherwise indicated. Data was analysed using GraphPad Prism v5.0.

Results

Galactosaminogalactan modulates human T-helper cytokine responses

To investigate whether GAG can exert immunomodulatory effects in humans, we tested whether GAG induces the production of pro- and/or anti-inflammatory cytokines in human PBMCs. GAG did not induce the pro-inflammatory cytokines TNFα, IL-6, IL-8, IFNγ, IL-17, IL-5 and IL-9 (Figure 1A), neither did it induce the anti-inflammatory cytokine IL-10 (Figure 1A). To determine whether GAG modulates *Aspergillus*-induced innate monocyte-derived cytokines, PBMCs were stimulated for 24

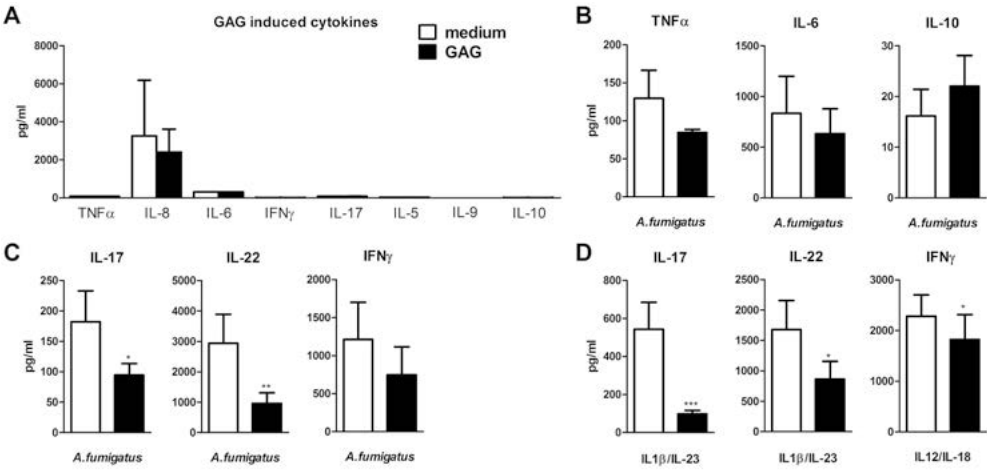

Figure 1 | GAG inhibits *Aspergillus*-induced human T-helper cell cytokine production
(A) TNFα, IL-6, IL-8 and IL-10 concentrations in culture supernatants of human PBMCs stimulated for 24hours with 10 µg/mL GAG and IFNγ, IL-17, IL-5 and IL-9 concentrations after 7 days of stimulation. (B) TNFα, IL-6 and IL-10 concentrations in culture supernatants of human PBMCs (n=6 donors) stimulated for 24 hours with heat inactivated *A. fumigatus* conidia (1x10^7/mL) in the presence or absence of 10 µg/mL GAG. (C,D) IL-17, IL-22 and IFNγ concentrations in culture supernatants of human PBMCs stimulated for 7 days with heat inactivated *A. fumigatus* conidia (1x10^7/mL) (n=10 donors for IL-17 and IL-22, n=6 donors for IFNγ) (c), IL-1β/IL-23 (50/100 ng/mL) (n=14 donors) or IL-12/IL-18 (50/100 ng/mL) (n=10 donors) in the presence or absence of GAG (10 µg/mL). Data are represented as mean ± SEM.

hours with *Aspergillus* conidia (these morphotypes of *A. fumigatus* were selected because they do not contain GAG that would interfere with the study of the immunological function of GAG) in the presence or absence of GAG. The presence of GAG did not have a significant effect on the production of the innate cytokines TNFα and IL-6, or the anti-inflammatory cytokine IL-10 (Figure 1B). However, when the production of the characteristic T-helper cytokines IL-17, IL-22 and IFNγ induced by *A. fumigatus* was investigated, the IL-17 and IL-22 responses were significantly reduced in the presence of GAG (Figure 1C). To determine whether the effects of GAG are specific for *Aspergillus*-driven T-helper (Th) responses, or whether GAG has a general ability to modulate human Th responses, the effects of GAG on cytokine-driven Th responses were studied. GAG significantly decreased the pro-inflammatory Th cytokine production induced by the cytokine combinations IL-1β/IL-23 and IL-12/IL-18 that induce IL-17/IL-22 and IFNγ, respectively (Figure 1E). Thus, GAG can inhibit human pro-inflammatory Th cytokine production induced by *Aspergillus* and cytokine cocktails.

Galactosaminogalactan induces IL-1 receptor antagonist

Human T-helper cytokine responses such as IL-17 and IL-22 production are highly dependent on the IL-1 receptor pathway [20,21]. To investigate whether the observed modulation of T-helper cytokines by GAG was due to an interaction of GAG with the IL-1 pathway, we determined the capacity of GAG conditioned medium (culture supernatants of PBMCs that were exposed to 10 μg/mL GAG for 24 hours) to reduce IL-1β bioactivity. Indeed it was shown that GAG significantly reduced the bioactivity of IL-1β while culture supernatants of unstimulated PBMCs did not (Figure 2A). Bioactivity of the IL-1 signalling pathway is dependent on IL-1 receptor agonists (IL-1α and IL-1β) and IL-1 receptor antagonists [22]. One of the natural inhibitors of the IL-1 signalling is the interleukin-1 receptor antagonist (IL-1Ra); therefore the ability of GAG to induce IL-1Ra was tested. IL-1Ra concentrations in the supernatant of the cells stimulated with GAG were significantly increased, whereas GAG induced none of the IL-1 receptor agonists, IL-1α or IL-1β (Figure 2B), showing that GAG has the capacity to modulate immune responses by blocking the IL-1 receptor pathway.

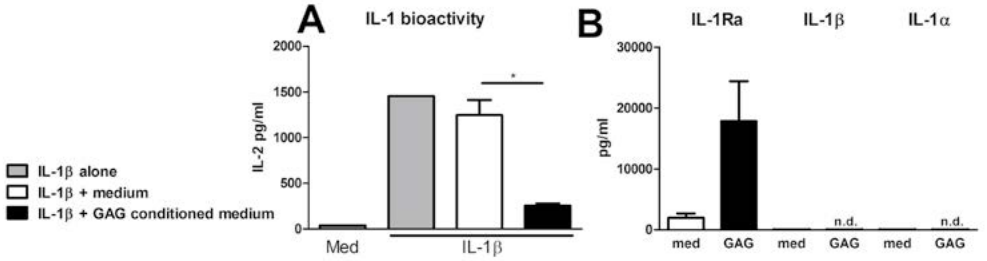

Figure 2 | GAG induces interleukin 1 receptor antagonist

(A) IL-1 bioactivity measured as IL-2 production by NOB-1 cells stimulated with 50 ng/mL IL-1β in the presence of culture supernatant of unstimulated PBMCs (medium) or culture supernatants of PBMCs that were exposed to 10 μg/mL GAG for 24 hours (GAG conditioned medium) (n=6 donors). (B) IL-1Ra, IL-1β and IL-1α concentrations in culture supernatants of human PBMCs stimulated with for 24 hours with 10 μg/mL GAG. Data are represented as mean ± SEM.

Suppression of IL-17 and IL-22 by galactosaminogalactan is dependent on IL-1Ra

To demonstrate that IL-17, IL-22, and IFNγ production by human PBMCs is indeed dependent on the IL-1 receptor pathway and that IL-1Ra can inhibit the production of these Th cytokines, Th1 and Th17 inducing stimuli were studied in the presence or absence of IL-1Ra. Addition of IL-1Ra reduced IL-17, IL-22, and IFNγ induction by both *Aspergillus* conidia and by IL-1/IL-23 and IL-12/IL-18 cytokine combinations (Figure 3A). To determine whether the immunosuppressive effect of GAG was mediated through the induction of IL-1Ra, PBMCs were stimulated with IL-1β/IL-23 and GAG in the presence or absence of neutralizing anti-IL-1Ra antibodies. GAG reduced IL-17 and IL-22 levels significantly, which was not observed in the presence of neutralizing anti-IL-1Ra antibodies, demonstrating that the inhibitory effects of GAG on Th cytokine production are dependent on IL-1Ra (Figure 3B).

Galactosaminogalactan induces IL-1Ra in vivo and IL-1Ra increases susceptibility to aspergillosis

The *in vitro* stimulations described above suggest that the immunomodulatory effects of GAG are due to inhibition of IL-1 bioactivity by inducing IL-1Ra. To assess whether this has relevant consequences *in vivo*, we measured IL-1Ra transcription in the lungs of mice infected with *Aspergillus* with or without the administration of GAG. Induction of *IL1RN* was increased in the presence of GAG (Figure 4A). To determine which cells are responsible for the induction of *IL1RN*, we isolated macrophages, neutrophils and epithelial cells from the lungs of naïve mice. Macrophages and neutrophils, but not epithelial cells, expressed *IL1RN* after stimulation with *Aspergillus* in the presence of GAG (Figure 4B). Interestingly, not all microbiological stimuli can prime for increased GAG-induced *IL1RN*, since pre-stimulation with LPS did not increase *IL1RN* induction by GAG (Figure 4B).

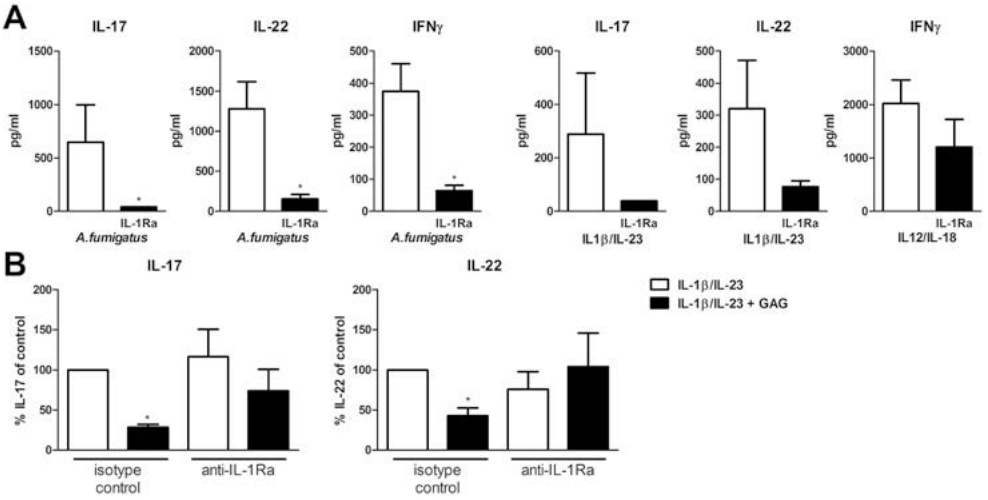

Figure 3 | Suppression of IL-17 and IL-22 by GAG is dependent on IL-1Ra

(A) IL-17, IL-22 and IFNγ concentrations in culture supernatants of PBMCs stimulated for 7 days with heat inactivated *A. fumigatus* conidia $1x10^7$/mL, IL-1β/IL-23 (50/100 ng/mL) or IL-12/IL-18 (50/100 ng/mL) in the presence or absence of recombinant human IL-1Ra (10 ng/mL). Data are represented as mean ± SEM. (B) Inhibition of IL-1β/IL-23 (50/100 ng/mL) induced IL-17 and IL-22 by GAG (10 μg/mL) in human PBMCs in the presence of isotype control (10 μg/mL) or anti-IL-1Ra (10 μg/mL). The IL-17 and IL-22 production by IL-1β/IL-23 in absence of GAG was set at 100% and mean percentage changes relative to the control are represented ± SEM.

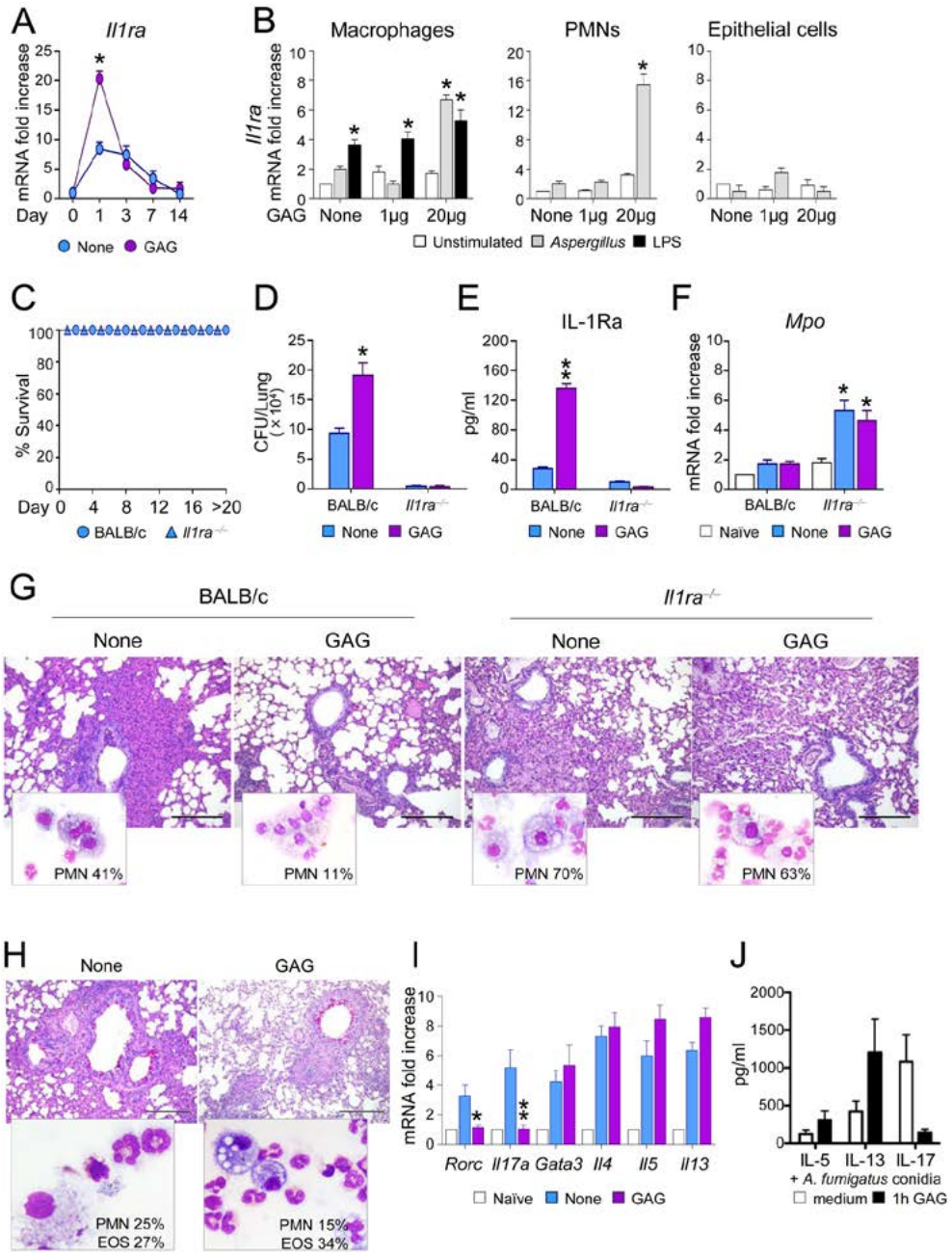

Figure 4 | GAG induces IL-1Ra *in vivo* and IL-1Ra increases susceptibility to aspergillosis
BALB/c and *IL1RN*$^{-/-}$ mice were intranasally infected with *Aspergillus* conidia and treated with GAG (250 µg/kg intranasally) the day of infection, and 1, 2 and 3 days post-infection. (A) *IL1RN* mRNA expression in lung homogenates of mice with invasive aspergillosis, (B) *IL1RN* mRNA expression in purified cells from lungs of naive mice pre-stimulated with *Aspergillus* conidia or LPS for 1 hour, and exposed to different GAG concentrations for an additional 18 hours. (C) Survival, (D) fungal growth (CFU/lung, mean ± SEM), (E) protein levels of IL-1Ra,

(figure 4 continued) (F) *Mpo* expression in lung homogenates, and (G) BAL morphometry [% polymorphonuclear (PMNs) cells and lung histology (PAS stained sections, bars indicate 20× magnification) of *Aspergillus*-infected mice with or without GAG treatment. Assays were done a day after the last GAG treatment. (H) BAL morphometry [% PMNs or eosinophils (Eo)] and lung histology (PAS stained sections, bars indicate 20× magnification), and (I) expression of Th transcription factors and cytokines in total cells from the draining lymph nodes in mice with ABPA and treated with or without GAG. Naïve means uninfected mice, and none means untreated mice and/or unstimulated cells. (J) IL-5, IL-13 and IL-17 concentrations in culture supernatants of PBMCs pre-incubated 1h either with medium or GAG (10 µg/mL). After washing, the cells were stimulated for 7 days with heat inactivated $1x10^7$/mL *A. fumigatus* conidia (n = 4 donors). Data are represented as mean ± SEM. *, $p<0.05$; **, $p<0.01$.

To investigate the significance of IL-1Ra *in vivo* and to determine whether the effects induced by GAG are dependent on IL-1Ra, we studied the effects of GAG in wild type (WT) and *IL1RN*$^{-/-}$ mice with invasive aspergillosis. *IL1RN*$^{-/-}$ mice were highly resistant to invasive aspergillosis, as indicated by long-term survival (Figure 4C) and reduced fungal burden (Figure 4D). Administration of GAG resulted in increased protein levels of IL-1Ra in the lungs of wild-type mice during infection (Figure 4E). In line with previous observations, GAG increased the susceptibility to invasive aspergillosis in WT mice but not in *IL1RN*$^{-/-}$ mice (Figure 4D). *IL1RN*$^{-/-}$ mice had increased *Mpo* expression (Figure 4F) and PMN influx in their respiratory tract (Figure 4G). As expected, administration of GAG reduced inflammatory PMN recruitment in WT but not in *IL1RN*$^{-/-}$ mice (Figure 4G). These data demonstrate that IL-1Ra has an important role in invasive aspergillosis, and support the concept that the induction of IL-1Ra by GAG may have important clinical consequences.

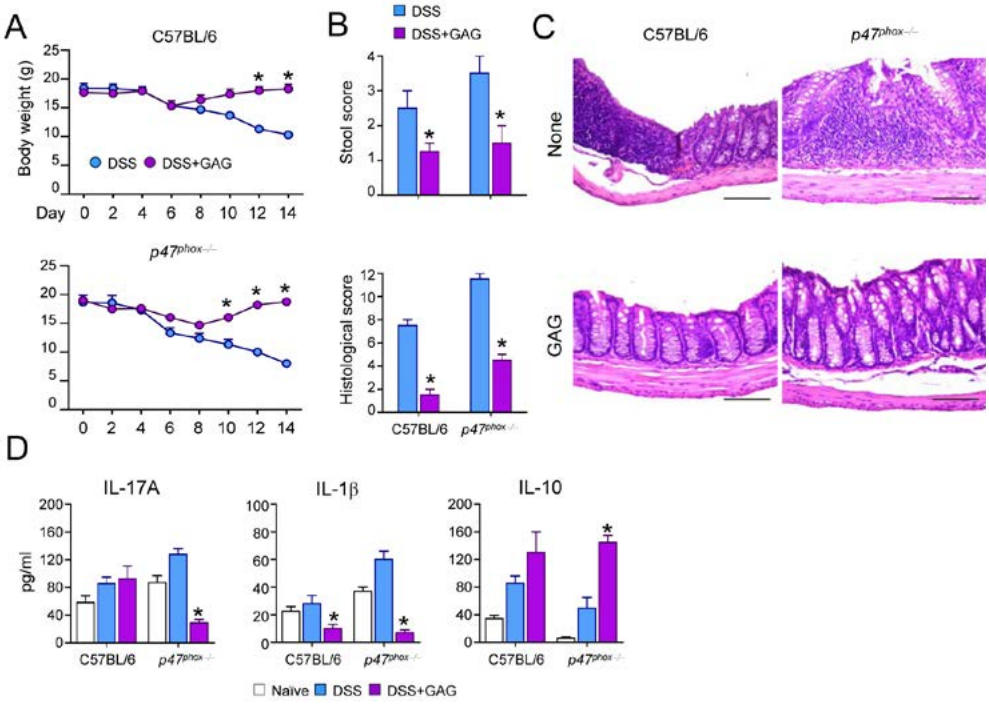

Figure 5 | GAG protects mice from experimental DSS-induced colitis
(A) Body weight losses, (B) stool and histological score, (C) histology of colonic sections and (D) cytokine concentrations present in total colonic cells a day after the 7-day of DSS rest in C57BL/6 and *p47*$^{phox-/-}$ (CGD) mice with or without GAG treatment. *$p<0.05$, GAG treated vs untreated mice.

In ABPA, GAG administration decreased PMN recruitment, but not eosinophilic infiltration in the BAL and lung of allergic mice (Figure 4H), a finding consistent with decreased Th17 but not Th2 cell responses in the draining lymph nodes (Figure 4I). To address whether GAG would have similar effects on human Th2 responses, PBMCs isolated form healthy subjects were pre-incubated for 1h with GAG and subsequently stimulated with *Aspergillus* conidia for 7 days. Similar to mice, IL-17 production decreased in the presence of GAG, but not Th2 cytokines such as IL-5 and IL-13 (Figure 4J). Thus, GAG has the potential to ameliorate Th17-dependent immunopathology in ABPA.

Galactosaminogalactan-induced IL-1Ra can be exploited as a therapy for IL-1-mediated inflammatory diseases

Since IL-1Ra treatment can be beneficial for autoinflammatory diseases such as chronic granulomatous disease CGD colitis in humans [23], we investigated whether GAG could be beneficial in experimental DSS-induced colitis in mice with chronic granulomatous disease (CGD). The administration of GAG resulted in the amelioration of clinical signs of colitis (weight loss and stool consistency) (Figure 5A-B) and of inflammatory lesions (Figure 5C) in both wild-type and CGD mice. However the protective effects of GAG were most apparent in CGD mice. GAG induced IL-1Ra and, consistently, reduced IL-1β and IL-17 (Figure 5D). Concomitantly, there was an increased production of IL-10, an anti-inflammatory cytokine that plays an important role in the protection of colitis [24,25]. The effects of GAG on CGD colitis were similar to those of IL-1Ra administration (unpublished data).

Discussion

In the original report describing GAG [7], it was shown that GAG has anti-inflammatory effects in mice. However, the mechanism through which GAG elicits its immunomodulatory effects remained a question at that time. In the present study, we demonstrate that GAG induces its anti-inflammatory effects by inducing the potent anti-inflammatory cytokine IL-1 receptor antagonist.

IL-1Ra can inhibit the activation of the IL-1 pathway by binding to the IL-1R type 1 receptor and prevents recruitment of the IL-1R accessory protein that is required for signalling. It has been repeatedly shown that IL-1 is an essential pro-inflammatory cytokine of the innate immunity. A deficient IL-1 pathway is also detrimental for the host, since it is an important protective pathway required to fight infection [26]. Thus the IL-1 axis is a potent pro-inflammatory pathway that needs to be tightly regulated, and IL-1Ra is a crucial player in this regulation. Therefore, it is rather surprising that the role of IL-1Ra in invasive fungal infection has not been studied in detail to date. We observed that the absence of IL-1Ra completely protects mice from developing invasive pulmonary aspergillosis, underscoring the importance of the IL-1 pathway in clearance of an acute invasive *Aspergillus* infection. The observation that GAG induces IL-1Ra *in vivo* identifies GAG as a potent anti-inflammatory molecule that suppresses the IL-1 pathway, subsequently resulting in increased susceptibility to invasive aspergillosis. The relevance of the IL-1 pathway in aspergillosis is underscored by the fact that polymorphisms IL-1 gene cluster polymorphisms are associated with susceptibility to develop in invasive pulmonary aspergillosis [27], and that dectin-1 knockout mice display increased fungal burden

and mortality during invasive aspergillosis, which is dependent on IL-1 [28].

One of the major risk factors that increases susceptibility to invasive aspergillosis is neutropenia [29], and neutrophils are crucial for clearing invasive germinating and hyphal forms of *Aspergillus* infection [30]. GAG has been shown to inhibit neutrophil recruitment to the lung, which is at least partly due to neutrophil apoptosis [7]. We observed that in the presence of GAG, IL-1Ra increased during invasive aspergillosis, which correlated with decreased PMN recruitment, and therefore increased fungal burden. In contrast, *IL1RN*$^{-/-}$ mice displayed increased neutrophil influx when exposed to *Aspergillus*, which could explain the resistance of *IL1RN*$^{-/-}$ mice to invasive aspergillosis, since they can rapidly and efficiently clear *Aspergillus* conidia due to their increased neutrophil response. In addition to the induction of IL-1Ra by GAG *in vitro* and *in vivo*, we observed that the inhibitory effects of GAG on the pro-inflammatory Th cytokine response in human PBMCs could be restored in the presence of a neutralizing antibody against human IL-1Ra. Furthermore, the increased susceptibility to invasive aspergillosis induced by GAG is not observed in *IL1RN*$^{-/-}$ mice. These observations strengthen the hypothesis that the anti-inflammatory properties of GAG are dependent on IL-1Ra.

The anti-inflammatory properties of GAG were present at a concentration of 10 µg/mL, which is a relevant concentration *in vivo*, since *Aspergillus* can secrete GAG in a concentration of 50 µg/mL (data not shown). The finding that antibodies against GAG are present in human serum [8] suggests that there is an adequate exposure of GAG to trigger the immune system. In the present study we were also able to demonstrate that these antibodies do not inhibit the effect of GAG, since we observed significant effects of GAG on IL-1Ra induction and inhibition of IL-17 in the presence of human serum that contained measurable concentrations of antibodies against GAG (data not shown). The relevance of GAG is highlighted by its presence in the extracellular matrix in aspergilloma resected from patients and mice with aspergillosis [8]. It is therefore expected that GAG plays a role in the immunological synapse between host immune cell and the mycelium, not only by inducing anti-inflammatory responses through IL-1Ra but also by shielding β-glucan from recognition, which has been proposed previously [9].

It must be taken into account that in the setting of chronic inflammation in which neutrophils and increased Th17 responses are detrimental for the host, IL-1Ra plays a protective role, due to its significant capability to suppress the IL-1 signalling pathway. This hypothesis is in line with the observation that *IL1RN*$^{-/-}$ mice develop spontaneous destructive arthritis that is IL-1 and Th17 dependent [31]. The importance of IL-1Ra in controlling IL-1-mediated pro-inflammatory responses in humans is underlined by a disease called deficiency of IL-1Ra (DIRA). This disease is characterized by the absence of IL-1Ra and severe Th17-mediated responses with neutrophil influx in the skin and bones of these patients, subsequently resulting in severe skin inflammation and osteomyelitis [32]. Therefore, the timing of IL-1Ra induction is of utmost importance to protect the host from infection and overwhelming inflammation. Chronic allergic aspergillosis is associated with excessive inflammation, with increased production of IL-1 and IL-22 [33]. We have demonstrated in the present study that administration of GAG induces IL-1Ra and is able to decrease IL-22 production. Therefore we investigated the effect of GAG in a murine model of allergic bronchopulmonary aspergillosis

(ABPA). We observed that the administration of GAG reduces the amount of neutrophils, but not eosinophils in ABPA. Additionally, Th17 responses were downregulated, but not Th2 responses. It is therefore tempting to speculate that administration of GAG can be beneficial in the setting of chronic allergic inflammation that is associated with excessive neutrophil-driven inflammation by reducing Th17 dependent pathology by inhibiting the IL-1 pathway. In addition, GAG also protected CGD mice from experimental colitis. Therefore, we envisage a model in which GAG on the one hand might be detrimental for the host in the setting of an acute infection, and on the other hand could be beneficial for the host during chronic inflammation driven by IL-1. Next to the identification of GAG or IL-1Ra as a therapeutic target for invasive aspergillosis, it is the first time that a polysaccharide produced by a human pathogen has been identified as an inducer of IL-1Ra by cells of the innate immunity without inducing pro-inflammatory responses, and which has been demonstrated to have therapeutic capacity in IL-1-mediated disease. The search of the sensing and signal transduction cascade activated by this polysaccharide will now be the center of future research.

The data presented here brings new questions into light and opens opportunities for future research. First, one of the most interesting observations is the complete protection of IL-1Ra knockout mice to invasive pulmonary aspergillosis. This opens new treatment strategies that target IL-1Ra in the setting of an acute invasive fungal infection. Second, the significant induction of IL-1Ra by GAG makes GAG or a derivative structure of GAG a potential treatment compound for IL-1-mediated diseases, such as joint, bone and muscle diseases and even very common inflammatory diseases such as diabetes and gout [34]. Previously, we have shown that mitogenic stimulation of monocyte derived macrophages and lymphocytes by αCD3/αCD28 coated beads, or recombinant cytokine-induced IL-17 and IFNγ production is inhibited in the presence of live *A. fumigatus* [35]. Although these changes in cytokine responses were attributed to changes in tryptophan and kynurenine, it is tempting to speculate that GAG secretion by live *A. fumigatus* could have attributed to the decreased IL-17 production.

In conclusion, our results demonstrate that GAG has potent anti-inflammatory effects in mice and humans that can be explained by the capability of GAG to induce IL-1Ra. These observations help to explain one of the immune-evasive mechanisms of *A. fumigatus*. Moreover, inhibition of GAG or IL-1Ra might prove beneficial in the treatment of acute invasive pulmonary aspergillosis, and GAG might be exploited for treatment of IL-1-mediated inflammatory diseases.

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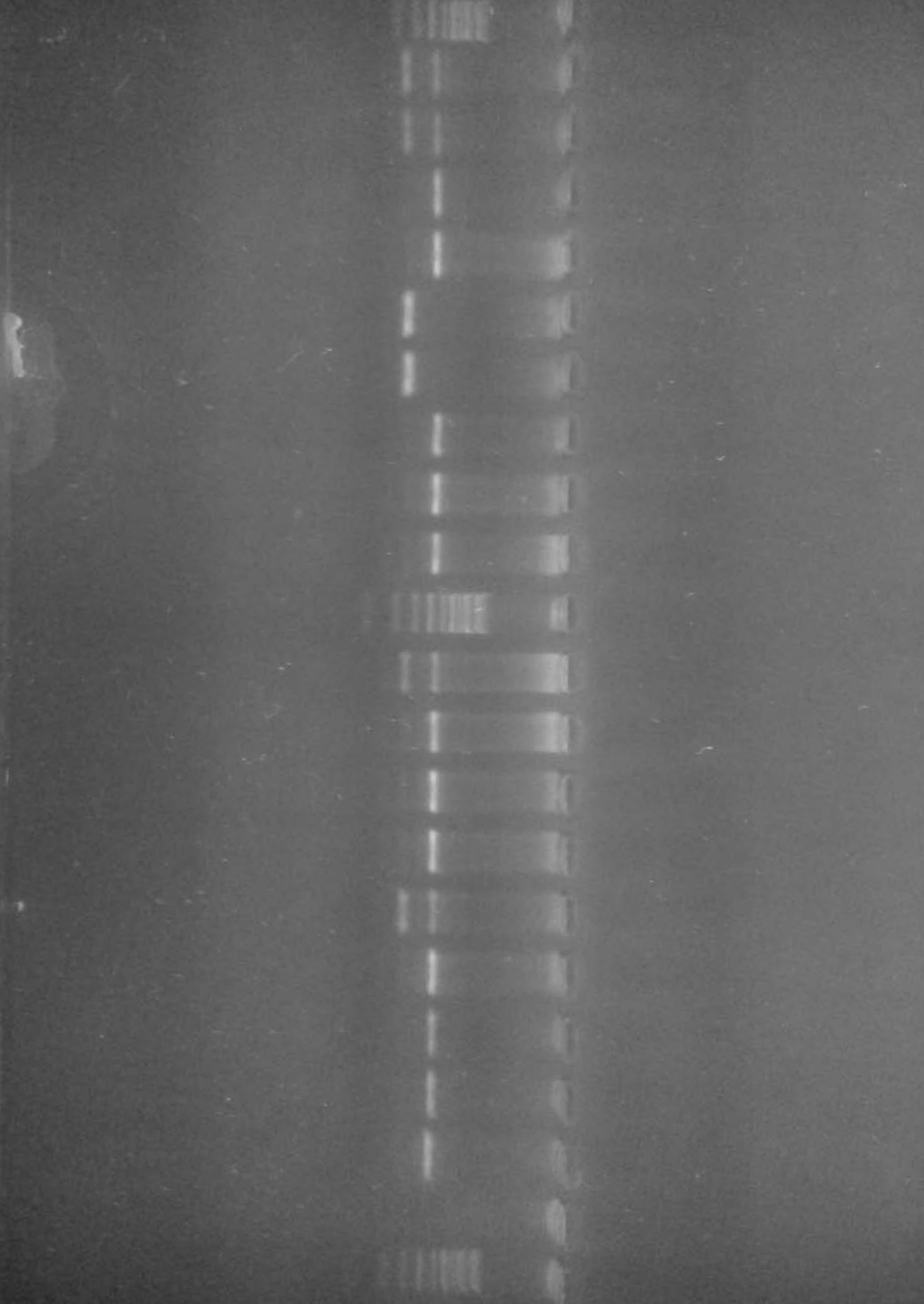

Chapter 9

IL1B and *DEFB1* polymorphisms increase susceptibility to invasive mould infection after solid organ transplantation

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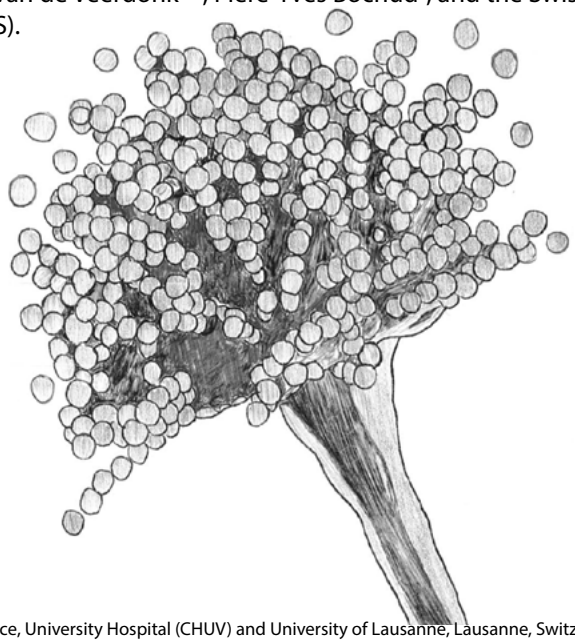

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Journal of infectious diseases,

Abstract

Background | Genetic polymorphisms have been associated with invasive mould infection (IMI) among hematopoietic stem cell transplant recipients. To our knowledge, no study explored the role of such polymorphisms among solid organ transplant (SOT) recipients.

Experimental procedures | The STCS is a large, nationwide, prospective cohort including all SOT recipients followed at six transplant centers in Switzerland. DNA was isolated from patient samples and 24 single nucleotide polymorphisms (SNPs) were genotyped using a custom-made Illumina® Golden Gate assay. Association between SNPs and the endpoint were assessed by log-ranktest and Cox regression model after adjustment for all relevant covariates. In PBMCs from healthy donors carrying SNPs associated with IMI in SOT patients, cytokine production upon *Aspergillus* stimulation was measured by ELISA and correlated to the genotype.

Results | The study included 1101 Caucasian SOT recipients (715 kidneys, 190 liver, 102 lungs,79 hearts, 15 other). Mould colonization (n=45) and IMI (n=26) were associated with polymorphisms in *IL1B* (interleukin-1β, rs16944; log rank test, colonization p=0.001 and IMI p=0.00005), *IL1RN* (interleukin-1 receptor antagonist; rs419598; p=0.01, and p=0.02) and *DEFB1* (β-defensin 1, rs1800972; p=0.001, and p=0.0002, respectively). The associations with *IL1B* and *DEFB1*, remained independently significant with IMI in a multivariate stepwise regression model (*IL1B* rs16944 p=0.0002, and *DEFB1* rs1800972 p=0.005). Presence of two copies of the rare allele of rs16944 or rs419598 was associated with reduced *Aspergillus*-induced IL-1β and TNFα secretion by PBMCs.

Conclusions | Functional polymorphisms in *IL1B* and *DEFB1* influence susceptibility to IMI in SOT recipients. This observation may contribute to individual risk stratification.

Introduction

Over 100'000 solid organ transplants (SOT) are performed worldwide each year [1]. Despite recent improvements in the management of SOT recipients, infectious complications after transplantation remain a challenging issue [2]. In particular invasive aspergillosis can occur in up to 3% of SOT recipients and is associated with a mortality rates ranging 20-76% [3,4]. Risk factors for the development of invasive aspergillosis include the type and level of immunosuppression, use of renal replacement therapy, older age, and CMV disease [3,5]. However, not all patients with these risk factors develop invasive mould infections (IMI), while some patients without these risk factors do, making it difficult to predict the risk to develop IMI at the individual level.

Over the last decade, a series of studies have identified common genetic polymorphisms that are associated with the development of invasive aspergillosis among hematopoietic stem cell transplant recipients and other onco-hematological patients [6,7]. The identification of specific genetic variants may improve individual risk stratification and allow the development of personalized management strategies, as well as to use prophylaxis or specific surveillance in individuals at high risk to develop invasive aspergillosis [6]. To date, no studies examined the role of such genetic polymorphisms on the susceptibility to fungal infections among SOT recipients. We explored for the first time the role of host genetics in susceptibility to IMI in a nationwide cohort of 1101 SOT recipients.

Experimental procedures

Patients and study design | The STCS is a large, nationwide, well documented prospective cohort including all SOT recipients followed at six Swiss University transplant centers (Basel, Bern, Geneva, Lausanne, St Gallen and Zurich) since May 2008 [8]. Patient data were systematically collected at enrollment, at six months and every 12 months after transplant on standardized case report forms.

Infectious complications were systematically evaluated by an infectious disease specialist based on clinical, histological, radiological and mycological evidence and reported on a separate case report form. Charts from patients reported to have fungal colonization or IMI were revised by an independent investigator (TL) [9]. Proven or probable IMI, was defined based on standardized EORTC/MSG guidelines definitions [10] and adapted ISHLT guidelines definitions unique for lung transplant recipients such as anastomotic bronchial infections or tracheobronchitis [11]. Colonization was defined by microscopic or culture detection of a mould from a specimen of a non-sterile site, including sputum, bronchoalveolar lavage, bronchial brush, sinus aspirate samples or urine in the absence of clinical signs/symptoms for infection. Patients who were diagnosed with mould colonization and/or IMI before transplant, and/or had received previous organ transplantation, were excluded. CMV infection was classified as asymptomatic replication, viral syndrome or probable and proven disease as previously reported [12].

Ethics statement | All patients provided written informed consent for participation to the STCS (including genetic analyses). The protocol was approved by the independent ethics Committees of each Swiss participating center (University Hospital of Lausanne (CHUV); University Hospitals

of Geneva (HUG); University Hospital Zürich (USZ); Cantonal Hospital St. Gallen (KSSG); Inselspital, Bern University Hospital; Clinica Luganese, Lugano; University Hospital of Basel). For functional assessment of polymorphisms, fresh venous blood was collected from healthy volunteers who provided written informed consent. The protocol was approved by the local ethics Committee (Radboud University Nijmegen Medical Center, The Netherlands).

PBMCs isolation and *in vitro* stimulation assays | Peripheral blood mononuclear cells **(**PBMCs) were isolated using Ficoll-Paque Plus (GE healthcare, Zeist, The Netherlands) density gradient centrifugation method as described previously [13]. Cells were subsequently stimulated with $1x10^7$/mL live or heat-inactivated *Aspergillus fumigatus* conidia for 24 hours or 7 days, respectively. Afterwards concentrations of the cytokines TNFα, IL-1β, IL-1Ra, IL-17, IL-22 (R&D systems, Minneapolis MN, USA) and IFNγ (Sanquin, Amsterdam, the Netherlands) were measured in cell supernatants by ELISA according to the manufacturer's protocol.

Genotyping | A total of 24 SNPs in 21 genes were selected from the literature (Table 2) by performing a PubMed search until June 2012 using the keywords: candidemia, candidiasis, aspergillosis, SNP and/or previous reviews on fungal immunogenetics [6,7]. Blood samples were obtained from all SOT recipients at the time of transplantation. Genomic DNAs was extracted from patients or healthy volunteer EDTA blood using the Gentra Puregene Blood Kit (Qiagen). Genotyping was performed using a customized GoldenGate Genotyping Assay on Veracode platform (Illumina, San Diego, CA, USA), unless otherwise indicated. Results were analyzed on a BeadXpress Reader according to standard protocols and quality controls. Additional SNPs were genotyped using Competitive Allele-Specific PCR (KASP) system (LGC Genomics, Herts, UK). For functional studies, *IL1B* rs16944 and *IL1RN* rs419598 variants were genotyped using pre-designed SNP assays on the ABI-Prism StepOne thermocycler (Applied Biosystems).

Statistical analysis | Statistical analyses were performed in Stata13 (StataCorp LP, College Station, Texas, USA), unless otherwise indicated. The cumulative incidence of mould colonization and IMI by genetic variants at 36 months after the first transplantation was assessed by the log-rank test, with censoring at the date of the last follow-up or death. Associated variants were selected based on the log-rank test and were further tested by uni- and multivariate Cox models. In order to estimate the independent contribution of each polymorphism to the endpoints, demographic and clinical factors previously associated with mould colonization and/or IMI (as described elsewhere [5,9]), were entered into multivariate stepwise regression models ($p<0.2$) together with relevant genetic polymorphisms. Haplotypes were inferred using PHASE 2.1 (University of Washington, Seattle, WA, USA). Power calculation for Cox proportional hazard regression was done using an R implementation of the power and sample size calculation for survival analysis of epidemiological studies (powerSurvEpi R package 0.0.6) [14]. The effect of presence of SNPs on the *Aspergillus*-induced cytokine levels was determined by the Mann-Whitney U test. The data are presented as mean ± standard error of the mean (SEM) and were analysed using Graphpad Prism v5.0 (San Diego, CA, USA).

Results

Cohort Study

The study included 1101 Caucasian patients who received solid organ transplantation (670 kidneys, 190 liver, 102 lungs, 79 hearts, 15 islets/pancreas and 45 combined organs) between May 2008 and December 2011 (Table 1). Mould colonization and IMI were diagnosed in 45 (4.1%) and 26 (2.4%) patients, respectively. Most IMI occurred >3 months after transplantation (n=17, 75%). The most frequent causative organism of IMI was *Aspergillus spp.* (n=21, 81%); only five IMIs were due to other fungi, including *Fusarium spp.* (n=2), *Altenaria spp.* (n=1), *Zygomycetes spp.* (n=1) and mixed pathogens (*Zygomycetes* and *Fusarium spp.,* n=1). Factors significantly associated with IMI were identified and described elsewhere [9] and included in the multivariate analysis.

Table 1 | Demographic characteristic of the solid organ transplant recipients

Variable	n	(%)
Recipient age (median years)	54	(19)
Donor age (median years) [1]	53	(22)
Recipient sex M/F	730 / 371	(66 / 34)
Donor sex M/F [2]	573 / 523	(52 / 48)
Duration of cold ischemia (median)	5.6	(7.3)
Transplanted organ		
Kidney [1]	670	(61)
Liver	190	(17)
Lung	102	(9)
Heart	79	(7)
Pancreas and islets	15	(1)
Mixed [3]	45	(4)
Donor type [4]		
Decreased	801	(73)
Living related/unrelated	299	(27)
Rejection type [5]		
Acute cellular rejection	342	(31)
Acute humoral rejection	35	(3)
CMV infection/disease	279/61	
CMV serostatus (n=1186) [6]		
D+ R+	350	(33)
D- R+	256	(24)
D- R-	245	(24)
D+ R-	218	(20)
Induction therapy [7]		
Basiliximab (BAS)	641	(61)
Anti-thymocyte globulin +/- BAS	188	(18)
None	225	(21)
Maintenance regiment [8]		
Calcineurin inhibitors	817	(92)
Corticosteroids	910	(83)
MMF	721	(81)
AZA	31	(3)
mTOR inhibitors	54	(6)
Anti IMI prophylaxis (weeks 1-4)	74	(7)

[1] Data were missing in 104 patients.
[2] Data were missing in 5 patients
[3] Including:
kidney-pancreas n=24
kidney-liver n=10
kidney-kidney n=5
kidney-islets n=3
kidney-kidney-pancreas n=2
kidney-lung n=1
[4] Data were missing in 2 patients
[5] Reported at any time of follow-up.
[6] Data were missing in 32 patients.
[7] Data were missing in 47 patients.
[8] Reported at month 12.

Abbreviations:
AZA: azathiopirine
BAS: Basiliximab
IMI: invasive mould infection
MMF: mycophenolate mofetil

Genetic risk factors for mould colonization and IMI in SOT patients

The minor allele frequency of the 21 SNPs are shown in Table 2. Three SNPs that deviated from Hardy-Weinberg equilibrium were excluded from the analyses. The power to detect mould colonization and IMI was calculated for each SNP (Table 4).

To assess the risk of fungal disease according to the different SNPs, we estimated the cumulative incidence of colonization and infection during the first 36 months after transplantation (Table 2). Mould colonization and IMI were both associated with SNPs in three different genes, including *IL1B* (rs16944 TT versus CT or CC; log-rank test p=0.001 and p=0.00005), β-defensin 1 (*DEFB1*, rs1800972 CC versus GG or CG, p=0.001 and p=0.0002) and the interleukin-1 receptor antagonist (*IL1RN,* rs419598 CC versus CT or TT, p=0.0 and p=0.02, respectively Figure 1). In addition, we observed a significant association between a SNP in surfactant-associated protein 2 and mould colonization (*SFTPA2*, rs17886395 GG versus CC or CG, log-rank test p=0.004) but not infection (p=0.5). However, this association was due to a small number of individuals (Figure S4).

Table 2 | Association of genetic variations with mould colonization or invasive mould infection

Gene (nt|aa change)[1]	Rs number	MAF	HWE	Mould colonization (n=45)[2] log-rank test p[3]	IMI (n=26)[2] log-rank test p[3]
IL1B (-511 C/T)	rs16944	0.33	0.160	0.001 *	0.00005 ***
DEFB1 (-44 C/G)	rs1800972	0.18	1.000	0.001 *	0.0002 **
IL1RN (2018T/C)	rs419598	0.25	0.320	0.01	0.02
TLR9 (-1237 C/T)	rs5743836	0.13	0.060	0.2	0.04
INFG (874 T/A)	rs2069705	0.33	0.086	0.05	0.06
PLG (D472N)	rs4252125	0.30	0.340	0.4	0.1
CD209 (-139 A/G)	rs2287886	0.36	0.610	0.2	0.2
IL10 (-1082 A/G)	rs1800896	0.44	0.220	0.8	0.2
TLR6 (S249P)	rs5743810	0.35	0.180	0.1	0.4
TNF (-308 G/A)	rs1800629	0.14	0.010	0.2	0.4
SFTPA2 (A91P)	rs17886395	0.14	0.900	0.004	0.5
CXCL10 (1642G/C)	rs3921	0.41	0.280	0.6	0.5
MBL (G54D)	rs1800450	0.15	0.099	0.8	0.4
IL10 (-819 C/T)	rs1800871	0.27	0.820	0.8	0.6
TLR1 (R80T)	rs5743611	0.07	0.500	0.6	0.7
TLR4 (D299G)	rs4986790	0.05	0.540	0.8	0.8
IL1A (-889 C/T)	rs1800587	0.29	0.620	0.6	0.8
MASP2 (D105G)	rs72550870	0.02	0.150	0.8	0.8
CLEC7A (Y238X)	rs16910526	0.08	0.340	0.7	0.8
TLR3 (L412F)	rs3775291	0.29	1.000	0.9	0.9
IL23R (R381Q)	rs11209026	0.07	0.811	0.7	0.7

[1] SNPs (*TLR1* rs5743618, *IL4* rs2243250 and *CARD9* rs10870077) deviated from Hardy-Weinberg equilibrium were excluded from the analyses.

[2] Five patients who were colonized with mould before transplant, among whom two also developed IMI before transplant, were removed from the analyses.

[3] p-value was assessed by log-rank test, recessive mode (patients homozygous for the rare alleles are compared to the other).

* Significant after correction for multiple testing (21 tests; p=0.02).

** Significant after correction for multiple testing (21 tests; p=0.004).

To determine whether the SNPs were independent risk factors for the mould colonization and IMI, we used multivariate Cox stepwise regression models, after adjustment for all relevant covariates (Table 3). The final model for colonization still included *IL1B* rs16944 (HR=2.52, CI 1.18-5.36, *p*=0.02), *DEFB1* rs1800972 (HR=6.11, CI 2.28-16.4, *p*=0.0003) and *IL1RN* rs419598 (HR=3.35, CI 1.31-8.58, *p*=0.01). The final model for IMI still included *IL1B* rs16944 (HR=4.29, CI 1.71-10.8, *p*=0.002), *DEFB1* rs1800972 (HR=4.73, CI 1.46-15.3, *p*=0.01), but not *IL1RN* rs419598. Associations were stronger when the SNPs were combined together (for *IL1B* rs16944 and *DEFB1* rs1800972, HR=4.94; CI 2.06-11.8; *p*=0.003; for *IL1B* rs16944 and *IL1RN* rs419598 HR=4.64, CI 1.92-11.2, *p*=0.0006; Supplementary Figure1). In order to account for a possible confounding role of antifungal prophylaxis, the analyses were repeated after removal of patients who received an anti-mould prophylaxis. The associations between the SNPs in *IL1B, DEFB1* and *IL1RN* were still significant (not shown).

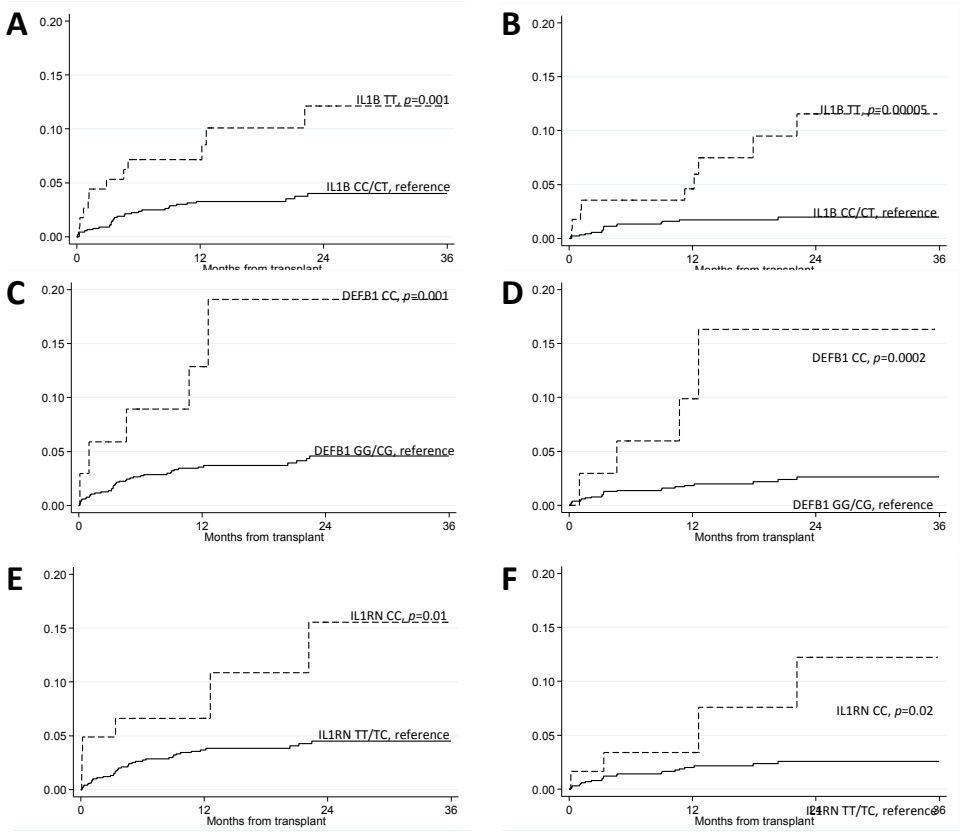

Figure 1 | Cumulative incidence of mould colonisation and invasive mould infection
(A) Mould colonization (n=45) and (B) invasive mould infection (n=26) according to *IL1B* rs16944 (panel A, and B, respectively), β-defensin 1 rs1800972 (*DEFB1*, panel C and D, respectively) and interleukin-1 receptor antagonist rs419598 (*IL1RN*, panel E, and F, respectively) polymorphisms in solid organ transplant recipients. Patients who were colonized or infected with mould before engraftment were excluded from the analyses. *p*-values were calculated by log-rank test, recessive mode (patients homozygous for the rate alleles are compared to the other). For *IL1B* rs16944 and *DEFB1* rs1800972 SNPs *p*-values remained significant after correction for multiple testing (21 tests; colonization for both polymorphisms *p*=0.02; infection *p*=0.001, and *p*=0.004, respectively).

***IL1B* rs16944 and *IL1RN* rs419598 risk haplotype for mould colonization and IMI**

IL1B and IL1RN genes are located within a ~400 kb region on chromosome 2q13-21, we therefore analyzed whether haplotypic combinations of *IL1B* rs16944 and *IL1RN* rs419598 SNPs further influenced mould colonization and IMI (Figure 2). Carriage of the rs16944-rs419598 C-T haplotype was associated with a decreased risk of both mould colonization and IMI (C-T haplotype vs. all other, HR=0.34, CI 0.18-0.63, *p*=0.0007 and HR=0.21, CI 0.10-0.45, *p*=0.00008, respectively). Reversely, carriage of the T-C haplotype was associated with an increased risk for both phenotypes (T-C haplotype vs. all other, HR=1.83, CI 1.02-3.29, *p*=0.04 and HR=2.09, CI 0.97-4.50, *p*=0.06, respectively).

Effect of *IL1B* rs16944 and *IL1RN* rs419598 polymorphisms on *Aspergillus*-induced cytokine release

In order to determine whether the SNPs associated with IMI and colonization had measurable biological effects, we analysed the production of different cytokines that are involved in antifungal host defence, including IL-1β, IL-1 receptor antagonist (IL-1Ra), TNFα, IL-17, IL-22and IFNγ in PBMCs from 73 healthy volunteers, after stimulation with live or heat-inactivated *A.fumigatus* conidia, respectively (Figure 2). PBMCs from volunteers carrying the *IL1B* rs16944 TT genotype produced lower amounts of IL-1β (*p*=0.01), TNFα (*p*=0.03) and IL-22 (*p*=0.03) after stimulation with *A. fumigatus,* compared to PBMCs from volunteers carrying the TC and CC genotypes. However, the production of IL-1Ra, IL-17 and IFNγ

Table 3 | Independent factors associated with mould colonization and infections in SOT recipients

Variable	Mould colonization (n=42) [1]		IMI (n=25) [1]	
	HR (95%CI)	*p* [2]	HR (95%CI)	*p* [2]
IL1B rs16944 (TT vs CC/CT) [3]	2.66 (1.25-5.64)	0.01	5.27 (2.19-12.7)	0.0002 **
DEFB1 rs1800972 (CC vs GG/GC) [3]	7.09 (2.62-19.2)	0.0001 *	5.34 (1.67-17.0)	0.005
IL1RN rs419598 (CC vs TT/TC) [3]	2.91 (1.13-7.47)	0.03	-	-
Transplanted Lung or Heart	7.60 (3.71-15.6)	<0.0001	3.45 (1.38-8.60)	0.008
MMF	0.31 (0.16-0.60)	0.0006	0.13 (0.06-0.30)	<0.0001
Tacrolimus	0.49 (0.25-0.98)	0.04	0.49 (0.20-1.15)	0.1
Corticosteroids	2.47 (0.71-8.61)	0.2	3.74 (0.82-17.1)	0.09
CMV infection/disease	-	-	2.75 (1.14-6.64)	0.02
Living donor	0.33 (0.07-1.47)	0.2	-	-
Recipient age (per year)	1.04 (1.01-1.06)	0.01	1.06 (1.02-1.10)	0.005

[1] The total number of patient in the multivariate analysis (n=1047) was slightly lower than in univariate analysis (n=1101) due to missing covariates. Five patients who were colonized with mould before transplant, among whom two also developed IMI before transplant, were removed from the analyses.

[2] Multivariate analysis assessed by stepwise regression. The variables included in the initial model were recipient age and sex, CMV infection or disease, mycophenolate mofetil treatment, acute cellular rejection, transplant from a living donor and type of transplanted organ.

[3] Genetic associations are for recessive mode (patients homozygous for the rare alleles are compared to the other).

* Significant after correction for multiple testing (n=21, *p*=0.002).

**Significant after correction for multiple testing (n=21, *p*=0.004).

Abbreviations: CI: confidence interval; CMV: cytomegalovirus; HR: hazard ratio; DEFB1: β-defensin 1; IL: interleukin; IL1RN: interleukin-1 receptor antagonist; IMI: invasive mould infection; MMF: mycophenolate mofetil.

was not significantly influenced by *IL1B* rs16944. PBMCs from volunteers carrying the *IL1RN* rs419598 CC genotype produced lower amounts of IL-1β (p=0.03) and TNFα (p=0.04) after stimulation with *A. fumigatus*, compared to PBMCs from volunteers carrying the TC and TT genotypes. However, the production of IL-1Ra itself as well as IL-22, IL-17 and IFNγ was not influenced by *IL1RN* rs419598.

Discussion

While a number of investigators have reported associations between genetic polymorphisms and susceptibility to invasive aspergillosis among onco-hematological patients [6,7], the role of such polymorphisms has not been studied among SOT recipients. We report for the first time an association between polymorphisms in *IL1B*, its antagonist *IL1RN*, and *DEFB1*, on susceptibility to IMI in this population.

The *IL1B* gene encodes for the cytokine IL-1β that is essential in host defense against *Aspergillus* infection [15]. IL-1β is a potent pro-inflammatory cytokine that recruits neutrophils to the lungs during infection, which are crucial for clearing *Aspergillus* [16]. Resting *Aspergillus* conidia in the respiratory epithelium are detected by alveolar macrophages and/or dendritic cells (DCs). These cells express a wide variety of pattern recognition receptors (PRRs), [17] that detect molecular patterns from the fungal cell wall (e.g. o-linked mannan, galactomannan and β-(1-3)-glucan) [7]. Whereas macrophages produce TNFα and IL-1β upon recognizing *Aspergillus* resulting in the recruitment of neutrophils and monocytes, activated DCs will migrate to lymph nodes to induce protective T-helper cell activity. IL-

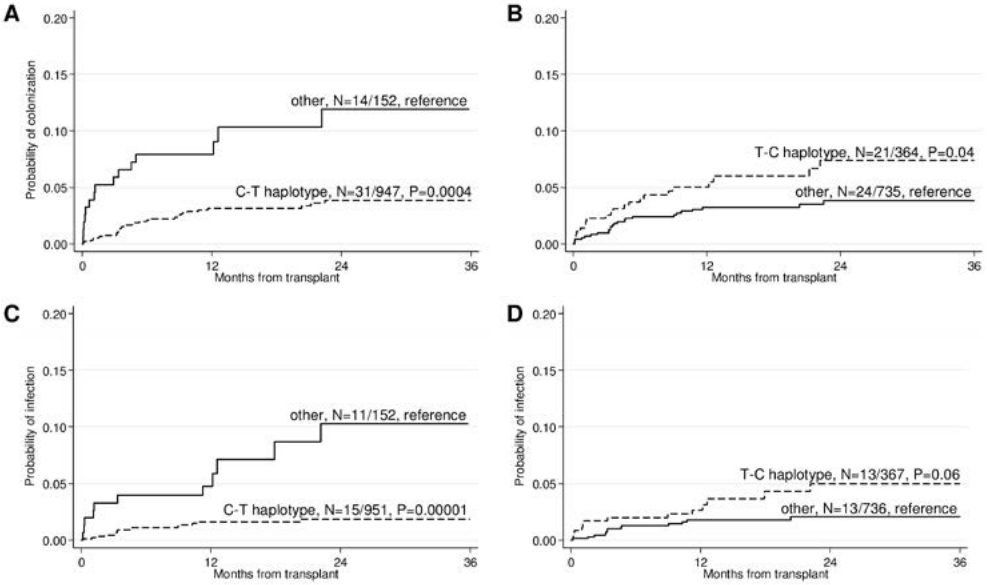

Figure 2 | Cumulative incidence of mould colonization and invasive infection in *IL1B-IL1RN* haplotypes (Panel A and B) and invasive mould infection (Panel C and D) according to rs16944-rs419598 C-T (Panel A and C) or T-C (Panel B and D) haplotype of *IL1B* and *IL1RN polymorphisms* in solid organ transplant recipients. *P*-values were calculated by the log-rank test by using the dominant mode of inheritance (patients carrying one or two copies of each haplotype are compared to the other). Patients who were colonized or infected with mould before engraftment were excluded from the analyses.

1β induces Th17 responses that are characterized by the production of IL-17, leading to an increased recruitment of neutrophils. Additionally, the activated T-helper cells induce IL-22 responses that will stimulate production of defensins by epithelial cells [7,13]. Thus, IL-1β is a key player in the induction of protective innate and adaptive anti-*Aspergillus* host defense.

Because of its potent inflammatory capacity, IL-1β responses need to be tightly controlled. This is underlined by the observation that patients with a mutation in IL-1Ra have severe inflammation of the skin and bones due to uncontrolled neutrophil influx and increased Th17 responses [18]. Moreover IL-1Ra knockout mice were shown to be fully protected from developing invasive aspergillosis [19]. Importantly galactosaminogalactan, an anti-inflammatory cell wall component of *A. fumigatus*, was able to induce IL-1Ra *in vivo* and consequently suppress the IL-1β pathway leading to increased susceptibility to invasive aspergillosis [19]. IL-1β binds to the IL-1 receptor and this results in the recruitment of a second receptor (IL-1RacP) [20] that activates signalling transduction pathways thereby exerting potent inflammatory activities. IL-1Ra also binds to the IL-1 receptor, but prevents recruitment of the second receptor and thus does not activate signal transduction pathways [20]. Therefore, the bioactivity of IL-1β is controlled by IL-1Ra [21].

The polymorphisms associated with mould colonization and IMI in this study are located within the IL-1 cluster, located in chromosome 2, encompassing both *IL1B* and *IL1RN*. We found that the minor alleles of rs16944 and rs1143627 within *IL1B* were associated with an increased risk of mould colonization and IMI in SOT. Consistent with our observation, the minor allele of rs16944 tended to be associated with an increased risk of invasive pulmonary aspergillosis in a case-control study of

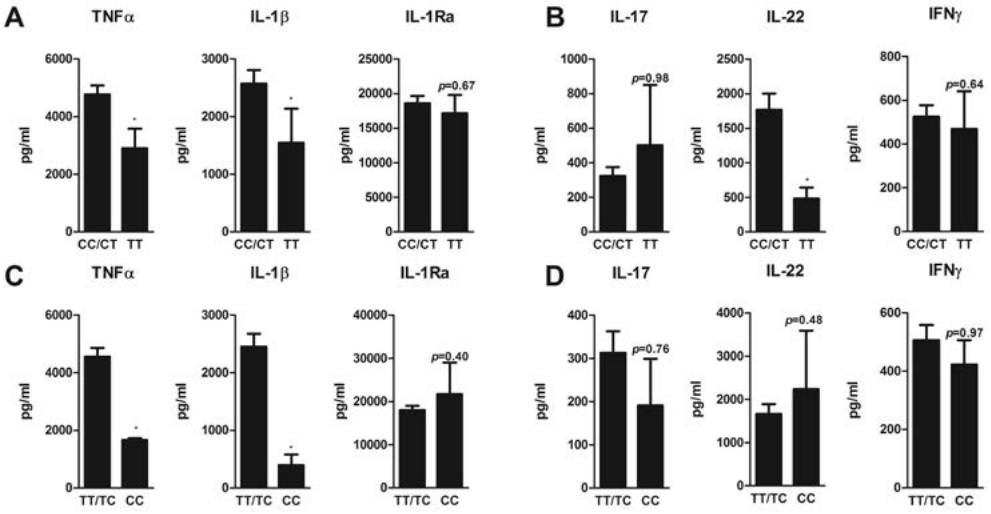

Figure 3 |Effect of *IL1B* and *IL1RN* polymorphisms on *Aspergillus*-induced cytokine release
Levels of *Aspergillus*-induced TNFα, IL-1β, IL-1Ra, IL-17, IL-22, IFNγ release by PBMCs depending on presence or absence of two copies of *IL1B* rs16944 (A) or *IL1RN* rs419598 (B) SNPs. PBMCs from 73 healthy volunteers with different *IL1B* rs16944 or *IL1RN* rs419598 genotypes were stimulated either with live *A. fumigatus* conidia for 24 hours (for TNFα, IL-1β, IL-1Ra) or heat inactivated *A. fumigatus* conidia for 7 days (for IL-17, IL-22, IFNγ). Cytokine levels were measured in cell culture supernatants and given in pg/mL. The effect of SNPs on cytokine levels were calculated by Mann-Whitney U test. The data are presented as mean ± standard error of the mean (SEM).

110 neutropenic patients with hematological malignancies [22]. The minor alleles of these SNPs were also associated with susceptibility to different bacterial and viral infections. Two studies showed an association between rs16944 and/or rs1143627 and mortality due to meningococcal disease [23]. Studies among Chinese patients also showed an association between both SNPs and susceptibility to sepsis after major trauma [24] and H1N1 pandemic influenza A virus [25].

It has been previously shown that rs16944 located at position -511 corresponding to a putative AP-2 binding site and rs1143627 located at position -31 within TATA box, are functional polymorphisms that could be responsible for alteration of promoter activity and thus being able to modulate expression and secretion of IL-1β *in vitro* [26]. In our study, PBMCs from individuals carrying two copies of rare allele of *IL1B* rs16944 had diminished IL-1β release upon *Aspergillus* stimulation. In line with our data, human monocytes from individuals carrying minor allele of rs1143627 had moderated transcriptional activity of *IL1B* promoter in response to lipopolysaccharide (LPS) [26]. Moreover carriers of minor alleles of rs16944 or rs1143627 had significantly lower IL-1β levels in LPS-induced PBMCs [27]. However, other studies have reported that the minor allele of rs16944 was correlated with increased transcriptional activity of the promoter *in vitro* upon LPS stimulation and high IL-1β secretion [28,29]. There is no clear explanation for these discrepant observations, but they may be due, at least in part, to the use of different cells or tissues and/or stimuli, stimulating concentrations or durations, or failure to account for other, so far unknown, regulating factors. We found that the *IL1B* rs16944 polymorphism was not only associated with less IL-1β production, but also reduced TNFα production in response to *Aspergillus.* TNFα is another essential cytokine in antifungal host defense, and anti-TNF treatment has previously been associated with invasive aspergillosis [24,30].

The *IL1RN* rs419598 SNP, which is in strong linkage disequilibrium with the *IL1RN* VNTR, has also been associated with mould colonization and IMI [31]. Consistent with this finding, the minor allele of rs419598 was previously associated with severity of meningococcal disease and genital human papillomavirus (HPV) clearance [23,32]. To our knowledge, the functional role of rs419598 has not been explored so far. In the present study, the minor allele of rs419598 did not influence IL-1Ra production in PBMCs from volunteers stimulated with *A. fumigatus*. However, rs419598 carriers had significantly lower levels of IL-1β and TNFα. This is consistent with a previous study, in which the *VNTR*2* was associated with decreased IL-1β in the gastric mucosa as well as in PBMCs stimulated with LPS [28,33]. Altogether, these data suggest that *IL1RN* participates to the regulation of *Aspergillus*-mediated cytokines production, and that *IL1RN* polymorphism may reduce the ability to clear the pathogen.

DEFB1 encodes human β-defensin 1, a member of the defensins family. Defensins are encoded by polymorphic gene cluster located within a 450-kb region on chromosome 8p23 [34]. They exert antimicrobial activity against a broad spectrum of pathogens; they also exert a chemotactic activity on immune cell and are able to induce cytokine production [35,36]. β-defensin 1 is constitutively expressed in lung epithelial cells and was shown to exert antimicrobial properties against *C. albicans* [37]. To our knowledge, the antimicrobial properties of this β-defensin against other fungal pathogens have not been investigated so far.

The minor allele of rs1800972 SNP in *DEFB1* was associated with an increased risk of mould

colonization and IMI in SOT. In line with our findings, the same allele was previously associated with higher oral *Candida* carriage among diabetic and non-diabetic patients [38]. The rs1800972 SNP is located at position -44 of 5′ untranslated region of *DEFB1* and was predicted to alter a putative transcription factor binding site to this region of the gene [39,40]. The minor allele of rs1800972 has been previously associated with lower β-defensin 1 expression in the skin with increased nasal carriage of *Staphylococcus aureus* [41]. This allele may also decrease the expression of β-defensin 1 in the respiratory epithelium, with reduced ability to clear opportunistic pathogens such as mould under certain immunosuppressive conditions. Interestingly, the cytokine IL-22 is a potent inducer of defensins produced by epithelial cells [42,43]. The *IL1B* rs16944 SNP was associated with a significant reduction in *Aspergillus*-induced IL-22 production in human PBMCs. Therefore, it can be proposed that this SNP may increase susceptibility not only because of the low production of essential innate cytokine such as IL-1β and TNFα, but also because of a reduced induction of IL-22 that might result in defective induction of defensins.

SFTPA2 encodes pulmonary surfactant-associated protein 2 (SP-A2), a member of collagen containing soluble CLRs (collectins). The SP-A2 was shown to play an important role in host response to *Aspergillus*, by its ability to bind *A. fumigatus* conidia and thus enhance phagocytosis by neutrophils and macrophages [44]. Polymorphisms in collagen region of *SFTPA2* (G1649C and/or A1660G) have been

Table 4 | Power to detect association of genetic polymorphism with mould colonization or invasive infection in solid organ transplant recipients.

Gene (nt|aa change) [1]	rs number	MAF	HWE	Colonization (Frequency 4.1%)				
				Power to detect colonization			Observed values	
				HR=3	HR=4	HR=5	HR	Cox p^2
IL10 (-1082 A/G)	rs1800896	0.44	0.220	0.62	0.90	0.98	0.89	0.8
CXCL10 (1642G/C)	rs3921	0.41	0.280	0.61	0.89	0.98	0.79	0.6
CD209 (-139 A/G)	rs2287886	0.36	0.610	0.58	0.87	0.97	0.50	0.2
TLR6 (S249P)	rs5743810	0.35	0.180	0.57	0.86	0.97	1.78	0.1
IL1B (-511 C/T) [4]	rs16944	0.33	0.160	0.55	0.85	0.96	2.98	0.002
INFG (874 T/A)	rs2069705	0.33	0.086	0.55	0.85	0.96	0.17	0.08
PLG (D472N)	rs4252125	0.30	0.340	0.51	0.82	0.95	1.45	0.4
IL1A (-889 C/T)	rs1800587	0.29	0.620	0.50	0.81	0.94	1.24	0.6
TLR3 (L412F)	rs3775291	0.29	1.000	0.50	0.81	0.94	1.10	0.8
IL10 (-819 C/T)	rs1800871	0.27	0.820	0.47	0.78	0.93	1.02	1.0
IL1RN (2018T/C)	rs419598	0.25	0.320	0.44	0.75	0.91	2.84	0.02
DEFB1 (-44 C/G)	rs1800972	0.18	1.000	0.30	0.59	0.79	4.08	0.003
MBL (G54D)	rs1800450	0.15	0.099	0.24	0.49	0.70	0.80	0.8
SFTPA2 (A91P)	rs17886395	0.14	0.900	0.22	0.45	0.66	4.79	0.009 [3]
TNF (-308 G/A)	rs1800629	0.14	0.010	0.22	0.45	0.66	0.00	1.0
TLR9 (-1237 C/T)	rs5743836	0.13	0.060	0.19	0.41	0.61	2.27	0.3
CLEC7A (Y238X)	rs16910526	0.08	0.340	0.09	0.21	0.34	0.00	1.0
TLR1 (R80T)	rs5743611	0.07	0.500	0.07	0.17	0.28	0.00	1.0
IL23R (R381Q)	rs11209026	0.07	0.811	0.07	0.17	0.28	0.00	1.0
TLR4 (D299G)	rs4986790	0.05	0.540	0.04	0.09	0.16	0.00	1.0
MASP2 (D105G)	rs72550870	0.02	0.150	0.01	0.02	0.03	0.00	1.0

associated with chronic cavitary pulmonary aspergillosis [45] and allergic bronchopulmonary aspergillosis in asthmatic patients [46]. Here, we detected an association between the G1649C polymorphism and mould colonization, but not infection, in SOT recipients. The lack of association with infection may be due to insufficient statistical power due to the relatively small SNPs allele frequency and sample size.

By using a list of 24 polymorphisms from 21 genes previously associated with susceptibility to fungal infections, we showed that SNPs from three genes (*IL1B*, *IL1RN* and *DEFB1*) were associated with mould colonization or IMI in Caucasian SOT recipients. Mould colonization may have been underestimated, as bronchoscopy is not routinely performed, especially in especially in non-thoracic SOT recipients. Yet, the polymorphisms associated with colonization were also associated with IMI, suggesting that these polymorphisms influence both phenotypes, with a *continuum* from colonization to invasive infection. We were not able to detect an association with polymorphisms in other genes, such as *TLR4* or *CLEC7A* (encoding Dectin-1), that were previously reported to be associated with invasive aspergillosis by several investigators [6,7]. This may be due to several reasons, such as the inclusion of different study populations, fungal pathogens and/or less invasive forms of infections. Importantly, the number of patients with mould colonization and/or IMI was small, leading to a limited statistical power, especially for infrequent SNPs, as detailed in Table 4. Larger studies and studies in patient from non-Caucasian ethnicities will be needed to replicate such associations.

Table 4 | continued)

IMI (Frequency 2.4%)				
Power to detect IMI			Observed values	
HR=3	HR=4	HR=5	HR	Cox *p* [2]
0.29	0.57	0.77	0.34	0.1
0.28	0.55	0.76	0.67	0.5
0.26	0.52	0.72	0.28	0.2
0.25	0.51	0.71	1.47	0.4
0.24	0.49	0.70	4.67	0.0002
0.24	0.49	0.70	0.00	1.0
0.22	0.46	0.66	2.24	0.1
0.21	0.45	0.65	0.81	0.8
0.21	0·45	0.65	0.94	0.9
0.20	0.42	0.62	0.56	0.6
0.18	0.39	0.58	3.36	0.03
0.12	0.27	0.42	5.91	0.001
0.09	0.21	0.34	0.00	1.0
0.08	0.19	0.31	0.00	1.0
0.08	0.19	0.31	0.00	1.0
0.07	0.17	0.28	4.13	0.054
0.03	0.08	0.13	0.00	1.0
0.03	0.06	0.11	0.00	1.0
0.03	0.06	0.11	0.00	1.0
0.02	0.04	0.06	0.00	1.0
0.005	0.009	0.01	0.00	1.0

[1] Three SNPs that deviated from Hardy-Weinberg equilibrium (*TLR1 rs5743618*, *IL4 rs2243250* and *CARD9 rs10870077*) were excluded from the analyses.

[2] P value was assessed by Cox regression, recessive mode (patients homozygous for the rare alleles are compared to the other).

[3] P value for the additive mode was 0.01, HR=1.91, CI 1.15-3.17

[4] In almost complete linkage disequilibrium (LD=0.99) with the IL1B -31T/C rs1143627

Abbreviations: CARD9: caspase recruitment domain-9; CI: confidence interval; CLEC7A: C-type lectin domain 7; CXCL10: CXC-chemokine ligand-10; CD209: CD209 molecule; DEFB1: β-defensin 1; HR: hazard ratio; IL: interleukin; IL1RN: interleukin-1 receptor antagonist; ; IL23R: interleukin 23 receptor; IMI: invasive mould infection; MBL: mannose banding lectin; MASP2: mannan-binding lectin serine peptidase 2; MAF: minor allele frequency; PLG: plasminogen; SFTPA2: surfactant protein A2; TLR: Toll-like receptor; TNF: tumour necrosis factor.

We report an association between polymorphisms in three genes and mould colonization and IMI in a nationwide cohort of SOT recipients. Theses associations were found to be independent from previously known risk factors, such as the recipient age, cytomegalovirus co-infection [3]. The genes were formerly described as important components of immune defenses against fungal pathogens, and the associated polymorphisms were all shown to be functionally relevant. Altogether, these findings may contribute to a better understanding of the pathogenesis of IMI in SOT recipients and help in individual risk stratification in the future.

Acknowledgements

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Supplementary material

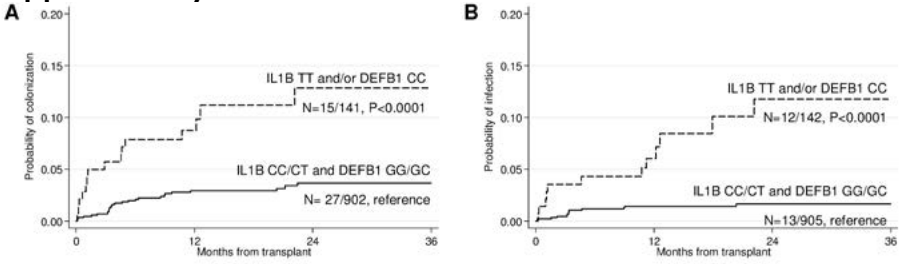


Figure S1 | Cumulative incidence of mould colonization and infection with *IL1B* and *DEFB1* genotype combinations

(Figure S1 continued) (A) Cumulative incidence of mould colonization and (B) invasive infection according to a combination of *IL1B* rs16944 with *DEFB1* rs1800972 polymorphisms in solid organ transplant recipients. Patients who were colonized or infected with mould before engraftment were excluded from the analyses. *p*-values were calculated by the log-rank test by using recessive mode of inheritance (patients homozygous for the rare alleles are compared to the other). Only one patient colonized and/or infected with the mould was homozygous for both *IL1B* rs16944 and *DEFB1* rs1800972 SNPs.

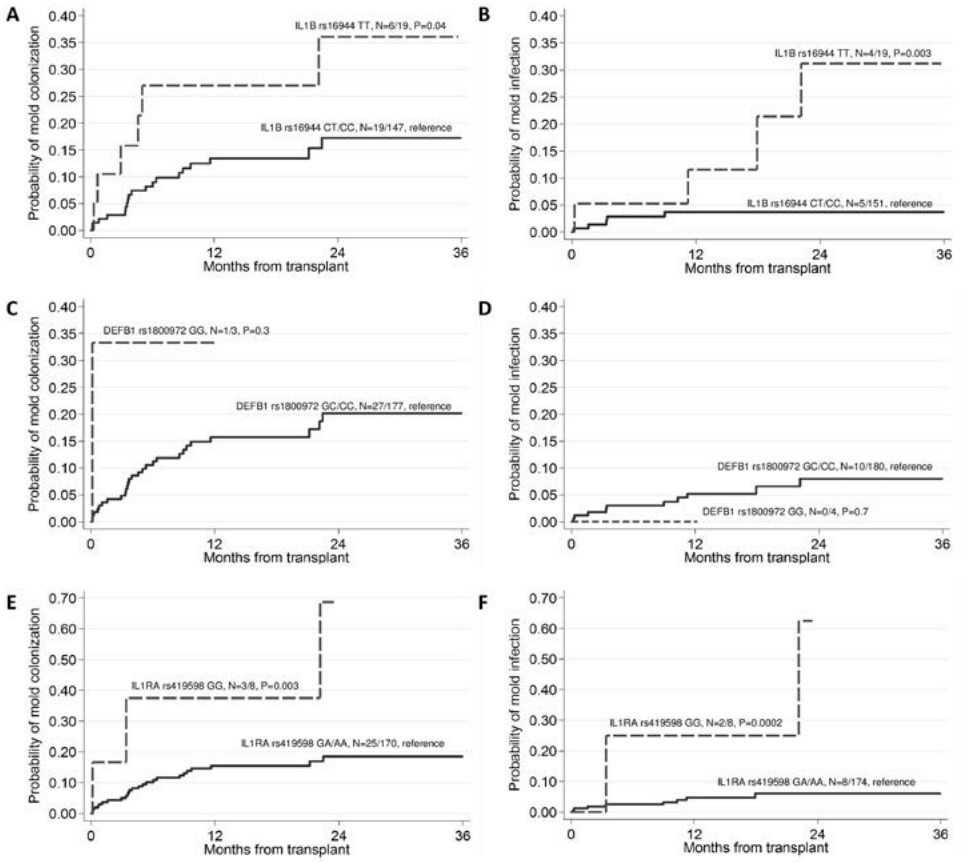


Figure S2 | Cumulative incidence of mould colonization and infection in thoracic solid organ transplant recipients

Cumulative incidence of mould colonization (n=25) and invasive mould infection (n=9) according to *IL1B* rs16944 (panel A, and B, respectively), *β-defensin 1* rs1800972 (*DEFB1*, panel C and D, respectively) and interleukin-1 receptor antagonist rs419598 (*IL1RN*, panel E, and F, respectively) polymorphisms in thoracic solid organ transplant recipients. Patients who were colonized or infected with mould before engraftment were excluded from the analyses. *P*-values were calculated by log-rank test, recessive mode (patients homozygous for the rate alleles are compared to the other)

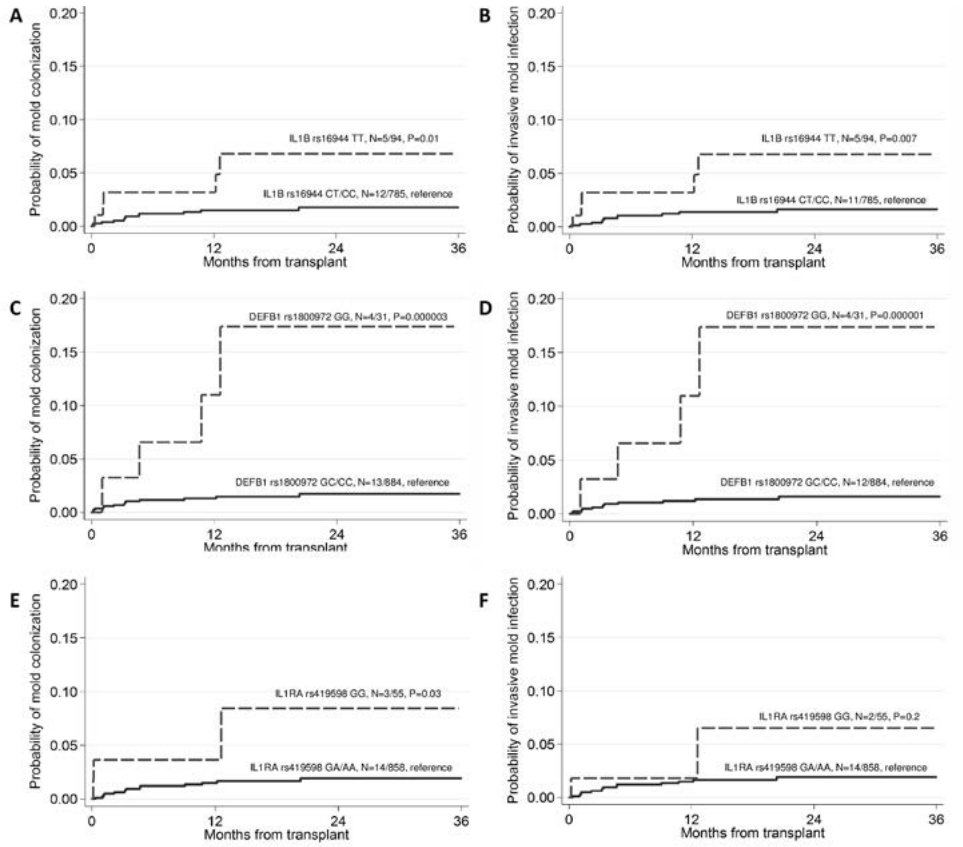


Figure S3 | Cumulative incidence of mould colonization and onfection in abdominal solid organ transplant recipients

Cumulative incidence of mould colonization (n=17) and invasive mould infection (n=16) according to *IL1B* rs16944 (panel A, and B, respectively), *β-defensin 1* rs1800972 (*DEFB1*, panel C and D, respectively) and interleukin-1 receptor antagonist rs419598 (*IL1RN*, panel E, and F, respectively) polymorphisms in abdominal solid organ transplant recipients. Patients who were colonized or infected with mould before engraftment were excluded from the analyses. *p*-values were calculated by log-rank test, recessive mode (patients homozygous for the rate alleles are compared to the other).

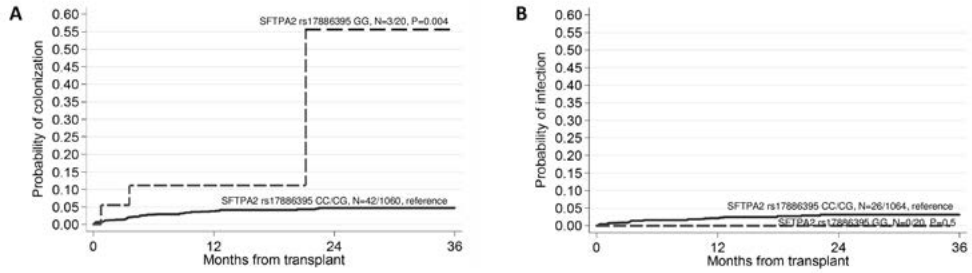


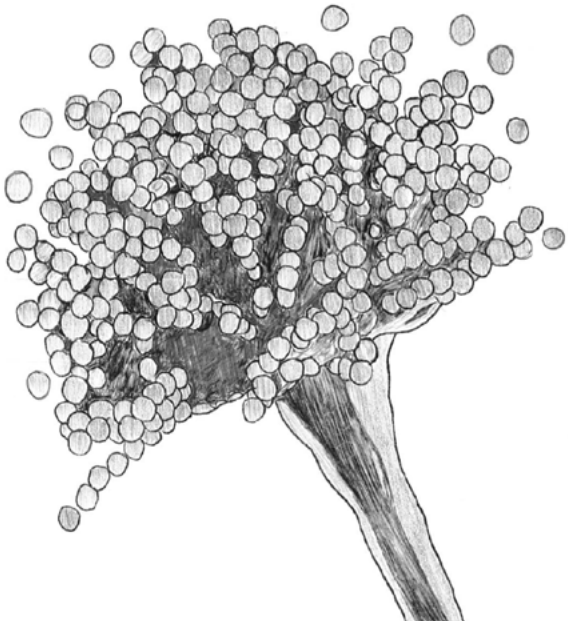
Figure S4 | Cumulative incidence of mould colonization and infection associated to *SFTPA2* genotype

Cumulative incidence of mould colonization (n=45) and invasive mould infection (n=26) according to *SFTPA2 rs17886395* (panel A, and B, respectively) polymorphism in solid organ transplant recipients. Patients who were colonized or infected with mould before engraftment were excluded from the analyses. *p*-values were calculated by log-rank test, recessive mode (patients homozygous for the rate alleles are compared to the other).

Chapter 10

Biology of IL-36 cytokines and their role in disease

Mark S. Gresnigt[1] and Frank L. van de Veerdonk[1,2]



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Abstract

IL-36α, IL-36β, IL-36γ, and IL-36Ra, collectively called IL-36 cytokines, are part of the IL-1 family. IL-36α, IL-36β, and IL-36γ are IL-36 receptor (IL-36R) agonists, while IL-36Ra is a receptor antagonist that blocks the activation of IL-36R signalling. IL-36 cytokines require processing in order to become fully active, however the protease(s) responsible for this are currently not known. The IL-36 receptor pathway activates dendritic cells and plays a role in polarizing T-helper responses. The skin is the predominant site where IL-36 cytokines are expressed and several reports have established that they play a significant role in the pathogenesis of skin diseases. In this review the discovery and biological function of the cytokines IL-36α, IL-36β, IL-36γ and IL-36Ra will be discussed, and their role in the pathogenesis of a wide variety of diseases.

Highlights

- IL-36 family cytokines share 20 to 52% homology with IL-1Ra.
- N-terminal processing is required for optimal bioactivity of IL-36 cytokines.
- IL-36 modulates immune responses by DC activation and T-helper subset polarization.
- IL-36 signalling plays a crucial role in skin homeostasis and inflammation.

Introduction

A decade ago several new members of the IL-1 gene family were discovered [1-9]. Two years after their discovery, these novel cytokines were renamed in order of their date of publication [10]. With the elucidation of their biological function it was proposed in 2010 that these IL-1 family members should each be assigned an individual interleukin designation [11]. The cytokines have been renamed: IL-36α, IL-36β, IL-36γ and IL-36 receptor antagonist (Ra), and are collectively called IL-36 cytokines (Table 1).

Discovery of the new members of the IL-1 gene family

IL-36Ra is the first new IL-1 family member that has been reported after the discovery of IL-1β, IL-1α, IL-1Ra and IL-18. IL-36Ra shares 52% homology with IL-1Ra and is predominantly expressed in the skin [1]. Shortly thereafter, another group reports that although IL-36Ra shares 47% homology with IL-1Ra, IL-36Ra does not have biological activity similar to IL-1Ra [2]. In addition to this IL-1Ra like molecule, three other IL-1 family members are described that share less homology to IL-1Ra [3,4]. One of these is IL-37, which will not be discussed in the present review. The other two, IL-36β and IL-36γ, share the core 12 fold, β-trefoil structure, and lack a signal peptide, classical features of IL-1 family members [3,4], but share 27 and 20% homology with IL-1Ra respectively [6,8]. IL-36Ra is constitutively expressed in keratinocytes, whereas IL-36γ expression in keratinocytes is rapidly induced after stimulation with TNF or PMA [3]. Smith *et al.*, identified IL-36α next to IL-36Ra, IL-36β and IL-36γ, and described the predicted three-dimensional structure of IL-36α [6]. Additional studies revealed that IL-36γ does not bind to the currently known IL-1 family receptors IL-1R, IL-18R or ST2 [4,6] However, in 2001 it was reported that IL-36γ can activate NFκB through IL-1Rrp2, and that IL-36Ra is able to block this activation, providing the first evidence that IL-36γ is an agonist and IL-36Ra is an antagonist [7]. IL-38 is the sixth newly discovered cytokine of the IL-1 family and shares 41% homology with IL-36Ra and 37% homology with IL-1Ra [9]. All six new IL-1 family member genes are located in a cluster on chromosome 2 and the gene order from centromere to telomere is *IL-1A-IL-1B-IL-37-IL-36G-IL-36A-IL-36B-IL-36RN-IL1F10-IL-1RN,* and only *IL-1A, IL-1B* and *IL-36B* are transcribed towards the centromere [8]. These new genes all map to a small interval, and they probably arose from a common ancestral gene, which is most likely a primordial IL-1 receptor antagonist gene [5].

Processing of the IL-36 cytokines

A specific feature of the IL-1 family cytokines is that they require N-terminal cleavage to process them into full bioactive cytokines. Recently, it has been reported that IL-36 cytokines like IL-1 and IL-18 also need to be processed in order to gain full bioactivity [12]. Truncation of IL-36α, IL-36β and IL-36γ results in a 1000-10.000 fold increased activity of these cytokines in comparison to their non-truncated forms. Interestingly, only when the N-terminal methionine of IL-36Ra is removed, IL-36Ra gains full antagonistic activity, which probably explains why initial studies do not show consistent results on the antagonistic property of IL-36Ra [7,12,13]. Proteases other than caspase-1 are likely to be responsible for cleaving these IL-36 cytokines, since the amino acids surrounding the truncation sites do not resemble a caspase-1 cleaving site [12]. Moreover, no proteolytic cleavage of IL-36α can

be observed in LPS/ATP-stimulated bone marrow derived macrophages with confirmed caspase-1 activity [14]. The protease(s) responsible for cleaving IL-36 cytokines are currently unknown.

Agonists of the IL-36 receptor pathway

IL-36γ is able to induce NFκB activation in Jurkat cells transfected with the IL-1Rrp2 receptor (IL-36R), and these observations provide the first line of evidence that these cytokines are agonists [7]. Furthermore, IL-36α, IL-36β and IL-36γ can all activate the MAPK, Erk1/2 and JNK through IL-36R/IL-1RAcP, which targets the IL-8 promoter and results in IL-6 secretion [13]. Additional studies have confirmed that IL-36α, IL-36β and IL-36γ all have agonistic characteristics and that they signal through the IL-36R [13,15,16]. In contrast to the other IL-1family cytokines the IL-36 cytokines are expressed in a more restricted manner. They are mainly expressed in keratinocytes, bronchial epithelium, brain tissue, and monocytes/macrophages (Table 1). In addition, T-lymphocytes can express IL-36α and IL-36β [6,17,18], and peripheral blood lymphocytes are able to express IL-36γ in response to α-particles, which can be used for targeted cancer therapy [19]. Interestingly, it has recently been shown that γδ T-cells can express IL-36β under specific conditions [20]. Interestingly, IL-36β can induce expression of itself, and thus has an autocrine/paracrine loop similar to IL-1 [21,22]. IL-17 and TNF can induce expression of IL-36α, IL-36β and IL-36γ in keratinocytes, which can be synergized by the cytokine IL-22 [21]. Furthermore, several reports indicate that epidermal growth factor signalling regulates the expression of IL-36α and IL-36β in the skin. Mice deficient in fibroblast growth factor receptors (FGFRs) have a defective

Table 1 | overview of the IL-36 cytokines

New nomenclature	Alternative names	Signalling	homology IL-1Ra	homology IL-1β
IL-36Ra	IL-1F5, IL1HY1, IL-1L1, IL-1RP3, IL-1H3, FIL1delta	Binds IL-1Rrp2 Blocks IL-1RAcP recruitment Recruitment of SIGIRR	52% 47%	26%
IL-36α	IL-1F6, FIL1epsilon		24%	30%
IL-36β	IL-1F8, IL-1H2, FIL1eta	Binds IL-1Rrp2 Recuitment of IL-1RAcP Activation of NFκB, MAPK, ERK1/2, JNK	27%	31%
IL-36γ	IL-1F9, IL-1RP2, IL-1H1		20%	31%

skin barrier that leads to activation of keratinocytes and γδ T-cells and significant expression of IL-36β in the epidermis [20]. Downstream of FGFR2 is ADAM17 (a disintegrin and metalloproteinase 17), and ADAM17 deficient mice show a similar skin phenotype to FGFR deficient mice and have an increased expression of IL-36α in the skin compared to wild-type mice [23]. These data point to an important role of the agonists IL-36α and IL-36β in skin homeostasis. Since the IL-36R is highly expressed in the brain, and more specifically on microglial cells and astrocytes, initial studies have investigated the role of these cytokines on brain inflammatory responses [24-26]. Murine IL-36 is expressed in neuron cells and in glial cells, but cannot be upregulated by LPS or IL-1β stimulation [24,26]. Furthermore, recombinant non-processed mouse IL-36β does not induce any of the classical IL-1 like responses in mixed glial cells in-vitro. Recombinant murine IL-36γ does not induce a response in mixed glial cells *in vitro*, and injection of IL-36 intra-cerebroventriclar does not result in fever or modification of food intake and body weight in mice [24]. Notably, these studies have been performed with non-processed IL-36 agonists, and therefore may not have revealed the genuine role of IL-36 cytokines in inflammatory responses in the brain. Bronchial epithelial cells express IL-36 cytokines, and stimulation of bronchial epithelial cells with pro-inflammatory cytokines such as IL-1+TNF, IL-17, *Pseudomonas aeroginosa* or TLR3 ligands results in significant expression of IL-36 [16,27]. In human lung fibroblasts, IL-36γ induces the chemokine IL-8 and the Th17 chemokine CCL20 [16]. These data suggest that IL-36 cytokines play a role in the host defence against viral and bacterial pulmonary infection and contribute to the regulation of neutrophil airway inflammation. Macrophages and monocytes express IL-36 [6,28]. Interestingly, THP-1

Processing for optimal bioactivity	expression	Induction	refs
Removal of N-terminal methionine (V2)	Monocytes, B lymphocytes, DCs , Keratinocytes, skin, Uterus, Placenta, Heart, Braim, Kidney		1-7, 11, 13, 15, 16, 63
Cleavage at 9 amino acids N-terminal to a conserved *A-X*-Asp (K6)	Monocytes, T/B-lymphocytes, Spleen, Bone-marrow, Tonsils, lymph nodes, Skin	IL-17, TNFα and IL-22 EGF	6, 7, 11, 13, 15-18, 21, 23
Cleavage at 9 amino acids N-terminal to a conserved *A-X*-Asp (R5)	Monocytes, T/B-lymphocytes, Bone-Marrow, Tonsils, Heart, Lung, Testis, Colon, neuron cells, glial cells	IL-36β IL-17, TNFα and IL-22 EGF IL-1β LPS	4, 6, 11, 13, 15-19, 21, 23, 24, 26
Cleavage at 9 amino acids N-terminal to a conserved *A-X*-Asp (S18)	peripheral blood lymphocytes, Keratinocytes, Bronchial epithelial cells, THP-1	IL-17, TNFα and IL-22 IL-1and TNFα IL-17 TLR3 *P. aeruginosa* *E. coli* LPS *P. gingivalis* LPS	3, 4, 11, 13, 15, 16, 19, 21, 16, 27

cells, a human monocyte cell line, specifically express IL-36γ, but not IL-36α or IL-36β, after stimulation with LPS derived from *E. coli* or *P. gingivalis* [28]. Although monocytes and macrophages can thus be a source of IL-36 cytokines, the significance of this observation with respect to homeostasis and pathogenesis remains to be elucidated.

IL-36 receptor antagonist

Although IL-36Ra shares the most homology with IL-1Ra, it significantly differs in loop conformations from IL-1Ra, most likely explaining the inability of IL-36Ra to bind to the IL-1R1. It has even been proposed that IL-36Ra is an agonist, since residue 147 is an aspartic acid in both IL- 1β and IL-36Ra, but a lysine in IL-1Ra; changing the lysine of IL-1Ra to an aspartic acid results in a partial agonistic character of IL-1Ra [29]. However, several reports have shown that IL-36Ra inhibits IL-36γ-induced NFκB activation [7,13]. Moreover, IL-36Ra acts similar to IL-1Ra, by eliciting its antagonistic effects through binding of the IL-36R and blocking the recruitment of the second receptor IL-1RAcP [12]. Although this suggests that IL-36Ra acts similar to IL-1Ra, several observations suggest otherwise. IL-36Ra needs to be processed in order to gain antagonistic properties [12]. Unlike IL-1Ra which to date was not found to induce any cytokines, IL-36Ra itself can induce mRNA of IL-4 and protein expression in glia cells in-vitro, and this induction is blocked in the presence of anti-SIGIRR antibodies, and in-vivo experiments show that the anti-inflammatory action of IL-36Ra in the brain is dependent on IL-4 and SIGIRR [30]. IL-36Ra reduces Th17 characteristic cytokines induced by *C. albicans* or *Aspergillus*, but not in a classical dose-dependent manner [31,32]. These reports suggest IL-36Ra might be able to activate an anti-inflammatory signalling pathway or recruit anti-inflammatory IL-1 orphan receptors like SIGIRR, and thus under specific conditions does not act as the classical receptor antagonist IL-1Ra.

IL-36 secretion

Because IL-36α, IL-36β and IL-36γ do not have a signal sequence they cannot be directed directly to the endoplasmatic reticulum, and thus require an alternative mechanisms that allows them to be secreted. The first study investigating the possible underlying mechanism of secretion of these IL-36 cytokines has studied the secretion of IL-36α in bone marrow-derived macrophages that overexpress IL-36α (BMDMs) [14]. IL-36α is secreted in these BMDMs upon stimulation with LPS/ATP, suggesting that IL-36α can be externalized in a stimulus-dependent manner comparable to IL-1β. Furthermore, they show that IL-36α has no caspase-1 cleavage consensus, and the release of IL-36α is not inhibited in the presence of a caspase-1 inhibitor [14]. In contrast, a recent study proposes that the release of IL-36γ by keratinocytes is caspase-1 dependent, and describes that IL-36γ transcription is dependent on caspase-1 [33]. It remains to be defined what the underlying mechanism is for secreting IL-36 cytokines.

The interplay of IL-36 cytokines with immune cells

The IL-36 cytokines have a significant effect on dendritic cells (DCs) and T-cells [18,34]. In murine DCs, IL-36 agonists upregulate CD80, CD86 and MHCII, markers of activation, and induce IL-6 and IL-12 production that is completely dependent on IL-36R signalling [18]. Although IL-36 cytokines do

not induce cytokine production in inactivated murine total splenic $CD4^+$ T-cells, in the presence of mitogenic stimulation (aCD3/aCD28), IL-36 agonists enhance the capability of CD4 T-cells to produce and secrete IFNγ, IL-4 and IL-17 in a dose dependent manner. In a murine immunization model using intradermal BSA injections, IL-36β can act as an adjuvant that specifically enhances Th1 responses in-vivo, and the adjuvanticity of IL-36β is completely dependent on IL-36R signalling [18]. In humans the expression of the IL-36R within the human monocytic cell line is unique to DCs [34]. Monocyte-derived DCs (MDDCs) and to a lesser extend plasmacytoid DCs express the IL-36R, while type 1 and type 2 DCs do not express IL-36R. Human MDDCs respond to IL-36β and IL-36γ by increasing their expression of HLA-DR and CD83. In addition, IL-36β induces the production of IL-12 and IL-18 in MDDCs, which subsequently results in the proliferation of IFNγ-producing T lymphocytes [34]. Stimulation of $CD11^+$ cells with IL-36α upregulates neutrophil chemokines CXCL1 and CXCL2, the cytokine TNFα and CD40 expression. Furthermore, these $CD11c^+$ cells were found to induce CD4 T-cell proliferation [35]. Although the IL-36R was initially found to be present on DCs, a recent study demonstrates that the IL-36R is also highly expressed on naïve T-cells [36]. IL-36 cytokines stimulate proliferation of T-cells and induce IL-2 production by naïve T-cells. Furthermore, IL-36 plays a significant role in the development of Th1 responses [36]. Human peripheral blood mononuclear cells that are stimulated with *C. albicans*, a potent inducer of the Th17 response in humans, produce less IL-17 and IL-22 in the presence of IL-36Ra [31]. The IL-36 receptor pathway also plays a role in regulating Th1 and Th17 responses induced by *A. fumigatus*, and neutralization of endogenous IL-36Ra could amplify the Th17 response to *Aspergillus* [32]. Furthermore, IL-36 agonists in the absence of mitogenic stimulation do not induce characteristic T-helper cell cytokines in mice [18] and humans [32]. Therefore, the data presented above suggest that IL-36 cytokines can modulate the immune system primarily through their effects on antigen-presenting cells, such as DCs and in this way can polarize T-helper responses. Furthermore, in the setting of activated T-helper cells, direct effects of IL-36 agonists have been observed and thus IL-36 cytokines can potentially play a role in fine-tuning T-helper responses during inflammation.

Role of IL-36 in disease

Since IL-36 cytokines are predominantly expressed in keratinocytes it is not surprising that specifically skin disorders have been explored for associations with these cytokines. Several reports show that psoriatic skin lesions express IL-36Ra and IL-36 agonists [7,37-39]. The importance of the IL-36 cytokines in regulating skin inflammation is underscored by the observation that transgenic mice which overexpress the IL-36α gene in basal keratinocytes display acanthosis and hyperkeratosis of the skin, which are characteristics of psoriatic skin lesions [38]. In addition, IL-36Ra deficiency exacerbated the skin lesions in IL-36α transgenic mice, suggesting an antagonistic effect of IL-36Ra *in vivo* for IL-36-mediated inflammation in the skin [38]. Phorbol ester treatment of mouse skin overexpressing IL-36α results in an inflammatory condition with macroscopic and histological similarities to human psoriasis [40]. In addition, the maintenance of characteristic inflammation of human psoriatic skin transplanted into immunodeficient mice is dependent on the IL-36R [40]. Psoriasis is associated with cytokines such as TNFα, IL-22 and IL-17, and the observed interplay between these cytokines and

IL-36 cytokines suggest an important role for IL-36 cytokines in psoriasis [21]. In line with this, anti-TNF treatment in patients with psoriasis results in decreased expression of the IL-36 agonists and IL-36Ra, which was associated with improved clinical outcome [37]. Although the expression of IL-36Ra by IL-17-stimulated keratinocytes derived from patients with psoriasis does not differ from healthy controls [41], increased expression of the IL-36 cytokines correlates with Th17 cytokines in human psoriatic skin lesions [21] and increased activation of MAPK and NFκB[39].

SNPs in a region in the IL-1 locus that includes the genes encoding IL-36β and IL-38 is associated with a higher susceptibility to psoriatic arthritis [42]. Moreover it has recently been shown that mutations in *IL-36RN* can cause a rare life threatening disease called general pustular psoriasis (GPP) [43-47]. The currently found mutations in *IL-36RN* lead to introduction of a premature stop-codon, frameshift mutation, or an amino acid substitution which were found to result in a misfolded IL-36Ra protein that is less stable and poorly expressed [43,45,46]., and Moreover the misfolded IL-36Ra has less affinity with the IL-36R compared to the wild-type IL-36Ra protein, and therefore is not able to dampen IL-36R-mediated inflammation [43,45,46]. These data indicate that IL-36Ra is a receptor antagonist, and that IL-36 signalling plays a significant role in regulating skin inflammation. Notably, mutations in the gene encoding IL-36Ra have not been found to date in Chinese patients with GPP, and only 2 out of 14 Japanese patients with GPP carried mutations in *IL-36RN* [46,48].

An early study reports that a polymorphism in IL-36Ra is associated with the development of alopecia areata, a disease characterized by patchy hair loss with T-cell infiltration in the hair follicles [49]. In contrast, a different SNP in IL-36Ra has been analysed in a large cohort of alopecia areata, which does not show an association. Another skin disorder where the IL-36R axis might play a significant role is Kindler syndrome, which is a rare syndrome characterized by skin blistering, increased photosensitivity of the skin, and progressive generalized poikiloderma [50]. IL-36Ra expression is increased in keratinocytes isolated from patients with Kindler syndrome [50]. Notably, these patients can also develop mucocutaneous fibrosis, such as stenosis of the esophagus and urethral stenosis. Additionally, psoriasiform dermatitis was found to be mediated by DC-keratinocyte cross talk independent on IL-1β, but dependent on IL-36 controlled IL-23, IL-17and IL-22 [51].

IL-36Ra is significantly upregulated in an animal model where brain micromotion is simulated [52]. Although LPS does not induce expression of IL-36Ra in microglia cells and astrocytes, IL-36Ra expression is increased by a 1000-fold in astrocytes and a 230-fold in microglia cells that are subjected to low-magnitude cyclical strain. Furthermore, primary cortical neurons that are stretched in the presence or absence of IL-36Ra, show a 400-fold decrease of TNF superfamily member B11 (TNFRSF11b) in the presence of IL-36Ra [52]. This decrease of TNFRSF11b is accompanied by a significant upregulation of pro-apototic genes, and suggests that IL-36Ra can regulate apoptotic pathways in low-magnitude strain induced brain tissue inflammation.

Preliminary data provide evidence that IL-36 cytokines might play a role in obesity. IL-36Ra is expressed in differentiated pre-adipocytes, and can be downregulated by the pro-inflammatory cytokine TNFα [53]. In addition, IL-36α, but not IL-36γ expression is present in adipose tissue-resident macrophages and its expression is induced by LPS. IL-36α and IL-36β can induce inflammatory gene

expression in mature adipocytes [54]. Furthermore, IL-1Ra plasma levels are associated with metabolic conditions and are under strong genetic control and interestingly; polymorphisms in IL-36β and IL-36Ra have been associated with plasma IL-1Ra levels in a cohort of 707 non-diabetic African Americans [55].

IL-36 might also play a significant role in joint disease. Remarkably, IL-36β is expressed in mouse and human joints [15] and IL-36α was found to be upregulated in joints of patients with psoriatic and rheumatoid arthritis [56]. IL-36β is constitutively expressed in human articular chondrocytes, and stimulation of both synovial fibroblasts and articular chondrocytes by recombinant IL-36β induces pro-inflammatory cytokine responses [15]. IL-36β can be measured in the serum of healthy human volunteers, but when serum IL-36β concentrations of healthy volunteers are compared to serum concentrations in rheumatoid arthritis no significant differences have been found [15]. In addition,

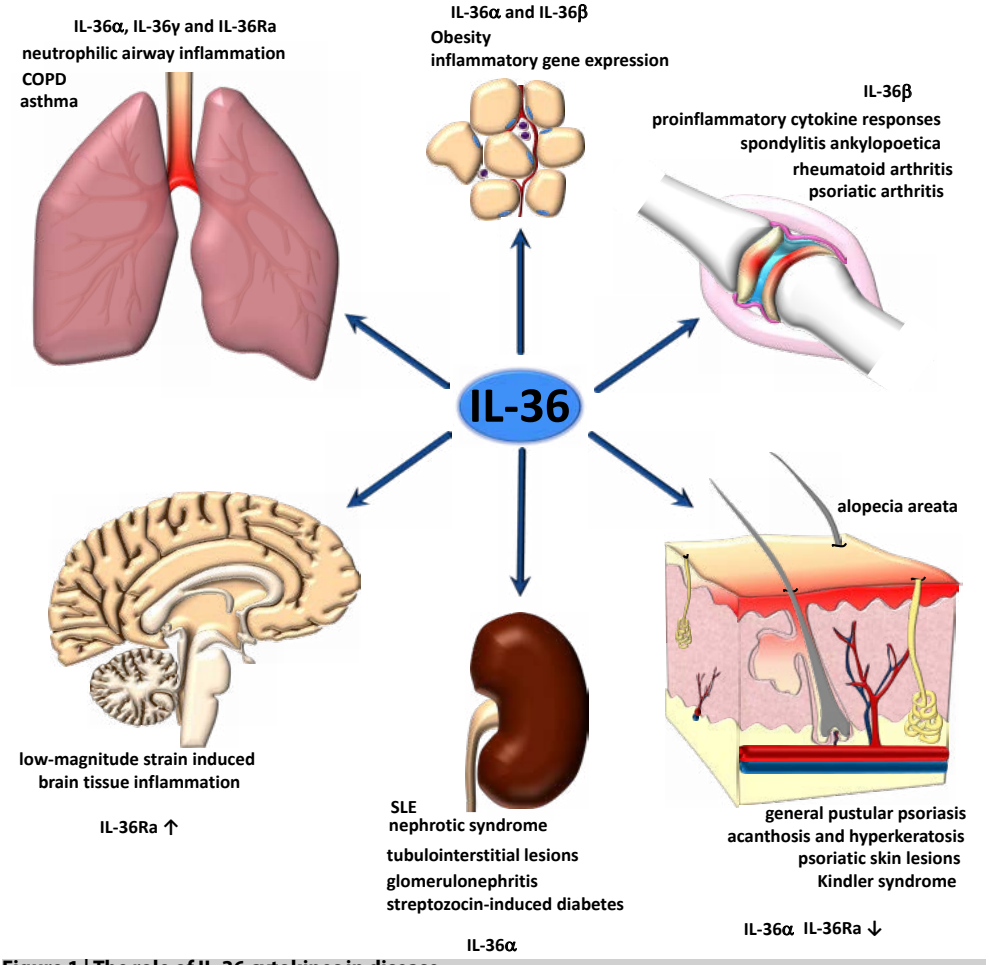

Figure 1 | The role of IL-36 cytokines in disease
Schematic overview of the organs and their specific diseases in which IL-36 cytokines have been found to play a role in disease pathogenesis.

polymorphisms in IL-36β are associated with spondylitis ankylopoetica in a Caucasian cohort, but not in Asian cohorts [57,58]. In contrast, recently it was demonstrated that in several experimental arthritis models IL-36γ, IL-36Ra and IL-36R were found to be expressed, but did not correlate with arthritis severity [59]. Furthermore, treatment with neutralizing antibodies against IL-36R did not affect arthritis severity in collagen induced arthritis.

The gene encoding IL-36γ is located in an allergen induced bronchial hyperresponsiveness-1 locus (*Abhr1*) in mice, and the expression of IL-36γ in the lung is increased in A/J mice (asthma susceptible) compared to C3H/HeJ mice (asthma resistant), suggesting that it might play a role in allergen-induced inflammation in the lung [60]. Indeed, in mice that are challenged with house dust mite (HDM), IL-36γ expression in the lung is significantly increased compared to non-challenged mice. It must however be noted that this difference is not explained by differential expression of IL-36γ by bronchial epithelial cells, and it remains to be elucidated which cell is responsible for the observed increased IL-36γ expression in the lungs of mice challenged with HDM. Recombinant IL-36γ given intratracheally, in a dose of 10 μg, results in neutrophil influx, but not eosinophilic influx in the lungs of mice, suggesting that IL-36γ is involved in the regulation of neutrophilic airway inflammation [61], which is in line with other studies [16]. Recently, it was found that IL-36α can induce neutrophil influx in the airways. Intratracheal administration of IL-36α resulted in upregulation of the neutrophil chemokines CXCL1 and CXCL2 and expression of the IL-36R in the lungs of both wildtype and IL-1αβ$^{-/-}$ mice. This supports the concept that IL-36 signalling can play a role in neutrophil recruitment independent of IL-1α or IL-1β [35]. Furthermore, rhinovirus-infected primary bronchial epithelial cells from patients with asthma show a higher expression of IL-36γ compared to infected cells from healthy controls [62]. A recent study that has investigated 507 inflammatory mediators in plasma from healthy volunteers, patients with COPD, and patients with COPD that have an acute exacerbation shows that 20 mediators are significantly different between these groups, of which IL-36α and IL-36Ra are significantly lower in patients with COPD that have an acute exacerbation [63]. These data collectively provide evidence that IL-36 cytokines play a significant role in regulating airway inflammation.

The role of IL-36 cytokines has also been studied in several other animal models of human disease. IL-36Ra is upregulated in a mouse model of biliary atresia [64]. Local overexpression of IL-36α in the kidney is associated with tubulointerstitial lesions in mouse models of chronic glomerulonephritis, SLE, nephrotic syndrome and streptozocin-induced diabetes [65]. However, the clinical significance of these finding remains to be elucidated.

IL-36 future issues

The IL-36R pathway shares features of the IL-1R1 pathway, however diseases such as deficiency of IL-1Ra (DIRA) and deficiency of IL-36Ra (DITRA) demonstrate that they each have unique non-redundant properties [43,66].

Furthermore, mice that overexpress IL-36α in basal keratinocytes have skin abnormalities that are dependent on IL-36R but not IL-1R1 [38]. Notably, the IL-36 cytokines are expressed in a more tissue-restricted way then IL-1. Therefore, IL-36 cytokines are not simple surrogates of IL-1; they are involved

in IL-1 independent inflammatory responses and they have most likely evolved to regulate tissue specific immune responses. However, the fact that a patients suffering from DITRA[67] or generalized pustular psoriasis due to mutations in IL-36Ra [68] can be treated with anakinra (recombinant human IL-1Ra), suggests that the IL-36 are controlled by classical IL-1 family cytokines.

To date, the IL-36 pathway has been associated with the pathogenesis of several inflammatory diseases. However, IL-36 pathway might also play a crucial role in antimicrobial host defence. For example, the host defence against fungi such as *C. albicans* and *A. fumigatus* can be modulated by the IL-36 receptor pathway [31,32]. In addition, Th1 responses to BCG are dependent on IL-36 cytokine signalling [36]. Yet the significance of the IL-36 pathway in antimicrobial host defence remains to be determined. With the current knowledge, it is tempting to speculate that IL-36 cytokines primarily play a role in skin and lung host defence.

An intriguing question that remains to be resolved is why IL-36Ra needs to be processed to gain fully antagonistic activity? Is it potentially dangerous to have IL-36Ra activity? Furthermore, what dramatic effects does the processing of the N-terminal methionine have on the molecule that it gains such increased activity? Moreover, which protease is responsible for this? The answers to these questions will probably further characterize IL-36Ra as an unique receptor antagonist that is different from the classical receptor antagonist IL-1Ra.

Another important issue is the presence of a unique mutation in the TIR domain of IL-36R (C445), which is only shared with the inhibitory IL-1 orphan receptor SIGIRR (C222) [69]. Studies investigating IL-36 biology have demonstrated that it has no consequence for agonistic activity of IL-36 ligands when IL-1RAcP is recruited to IL-36R [12]. This is elegantly proven by the fact that the chimera of the extracellular IL-1R1 with the intracellular IL-36R that has the mutated TIR domain does not result in an altered agonistic activity of IL-1 [12]. However, it is remarkable that this mutation exists in the TIR domain of IL-36R and it remains to be determined whether it has functional consequences in other specific conditions.

In conclusion, over recent years much has been learned about the IL-36 cytokines and their role in inflammation. They predominantly play a role in regulating skin inflammation, and they might have important modulatory functions in inflammatory conditions of the lung and the brain. This knowledge provides a rationale to further explore immune-modulatory strategies that target the IL-36 signalling pathway in the inflammatory conditions described in this review.

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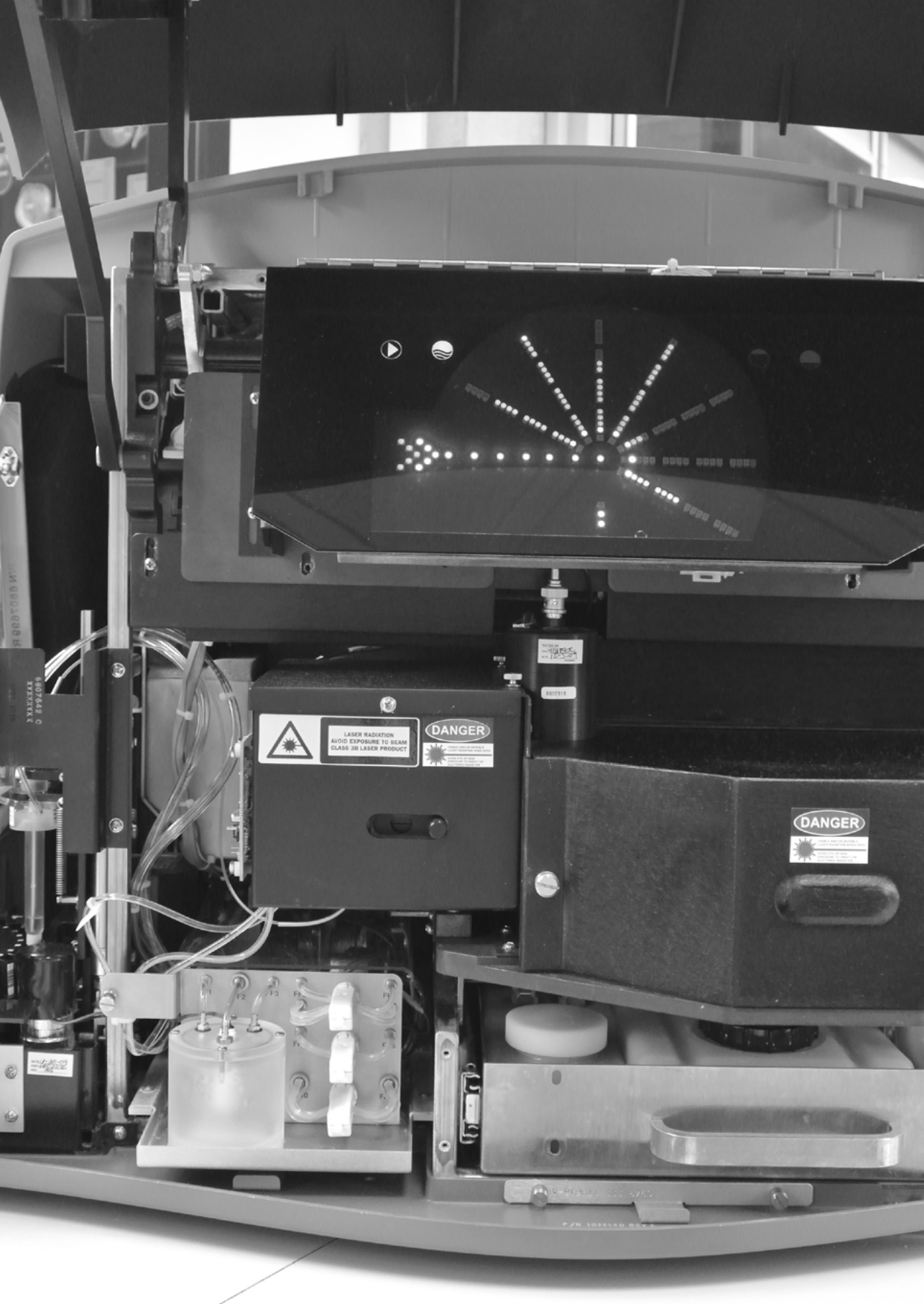
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Chapter 11

The interleukin-36 receptor pathway regulates *Aspergillus fumigatus*-induced Th1 and Th17 responses

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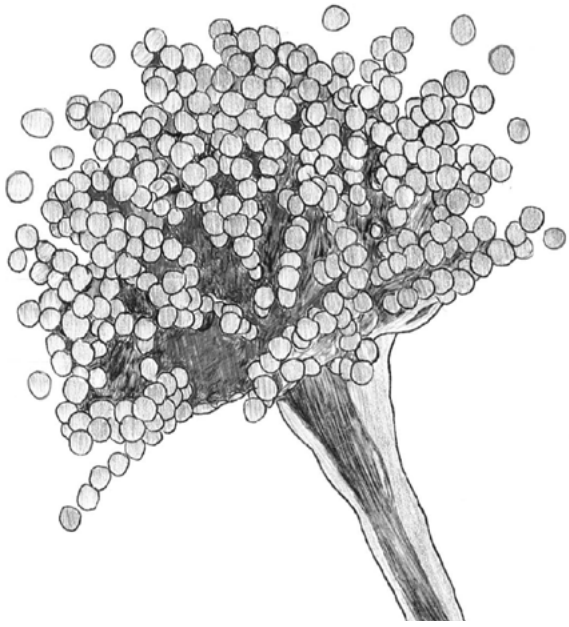

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Abstract

IL-1 drives T-helper (Th) responses, particularly Th17, in host defense. Sharing the same co-receptor, the IL-1 family member IL-36 exhibits properties similar to those of IL-1. In the present study, we investigated the role of IL-36 in *Aspergillus fumigatus*-induced human T-helper cell responses. We observed that different morphological forms of *A. fumigatus* variably increase steady-state mRNA IL-36 subfamily members. Although, IL-36α is not significantly induced by any morphological form of *Aspergillus*, IL-36β and IL-36 receptor antagonist (IL-36Ra) are induced by heat-inactivated resting conidia. Most strikingly, IL-36γ was significantly induced by live *A. fumigatus* conidia and heat killed hyphae, whereas IL-36Ra was significantly induced by heat killed conidia and hyphae and live conida. We also observed that IL-36γ expression is dependent on the dectin-1/ spleen tyrosine kinase (Syk) and TLR4 signalling pathway. In contrast, TLR2 and CR3 inhibit IL-36γ expression. The biological relevance of IL-36 induction by *Aspergillus* is demonstrated by experiments showing that inhibition of the IL-36 receptor by IL-36Ra reduces *Aspergillus*-induced IL-17 and IFNγ. These data describe that IL-36-dependent signals are a novel cytokine pathway that regulates T-helper responses induced by *A. fumigatus,* and demonstrate a role for TLR4 and dectin-1 in the induction of IL-36γ.

Introduction

The interleukin 1 family (IL-1F) consists of eleven members [1]. The in silico discovery of IL-1 family members IL-1F5, IL-1F6, IL-1F8, and IL-1F9 have recently been renamed IL-36 receptor antagonist (IL-36Ra), IL-36α, IL-36β, and IL-36γ, respectively. Presently, there is a paucity of data on the biological properties and functional roles of the IL-36 subfamily in health and disease. The classic IL-1 family members IL-1α and IL-1β as well as IL-18 and IL-33 polarize T-helper (Th) responses [1]. However, little is known about the role of the IL-36 subfamily (IL-36α, IL-36β, and IL-36γ) and the IL-36Ra in antimicrobial host defence. Each member of the IL-36 subfamily binds to IL-1Rrp2 (now renamed IL-36 receptor, IL-36R) and recruits the co-receptor for signal transduction, IL-1RAcP [2,3]. The IL-36R is expressed on dendritic cells (DCs) [4,5] and T cells [5]. Stimulation of T cells by IL-36R ligands results in the induction of IFNγ, IL-17 and IL-4 [5], and DCs primed with IL-36 ligands are inducers of Th1 cells. Although the synthetic TLR3 ligand polyI:C induces IL-36γ secretion [6], nothing is known about the innate pattern recognition pathways that induce IL-36R ligands in the antimicrobial host defence.

The opportunistic fungus *Aspergillus fumigatus* is ubiquitous; *Aspergillus* spores are inhaled daily by humans. Inhaled *Aspergillus* spores (conidia) are efficiently cleared in healthy individuals [7]. However, in immunocompromised persons, or in patients with pre-existing lung injury, *Aspergillus* conidia germinate resulting in invasive infections associated with high mortality [8]. T-helper responses are important in the host defence against invasive aspergillosis [9], and the major known cytokine pathways that are involved in the induction of Th1 and Th17 responses are IL-12/IL-18 and IL-1/IL-23 respectively [9,10]. Next to the induction of the specific Th-axis, there is also interplay between these cytokine pathways. IFNγ-induced IL-18 binding protein was found to downregulate the Th17 response [11], and in several cases the induction of Th17 or IL-17 producing γδT cells was found to be dependent on IL-18 [12,13]

In the present study, we have focused on gene expression of the IL-36 subfamily by different morphological phenotypes of *A. fumigatus.* In addition, we studied the IL-36R pathway in the induction of pro-inflammatory T-helper responses induced by *A. fumigatus.* Furthermore, we aimed to identify which pattern recognition pathways are involved in the induction of IL-36γ during *Aspergillus*-specific host defence. Here we demonstrate that the IL-36R pathway is involved in the regulation of *Aspergillus*-induced pro-inflammatory T-helper cytokine responses, and that the most important pathways for the induction of IL-36γ in *Aspergillus* host defence are represented by the dectin-1/ spleen tyrosine kinase (Syk) and TLR4 pathway.

Experimental procedures

Aspergillus fumigatus | A clinical isolate of *Aspergillus fumigatus* V05-27, which has been previously characterized [14], was used for all stimulations. Conidia and hyphae were prepared as described previously [15],the hyphae were quantified as the number of conidia used to grow the hyphae, and all hyphae were prepared in one batch to account for batch to batch variations. Aliquots from both the conidia and the hyphae were heat inactivated (HI) at 56°C for 1 hour and were checked

for viability in Sabouraud glucose broth. HI conidia, hyphae, and live conidia were stored at -80°C until use. A concentration of $1x10^7$/mL was used in the experiments, unless otherwise indicated. To study germination of live *A. fumigatus* conidia, $1x10^7$conidia/mL were plated in 12-wells plates. At 0, 2, 4 and 8 hours, a 10 µL sample was taken and observed and photographed using a brightfield microscope at 1000x magnification (Leica DM 3000).

Cytokines | Recombinant, IL-1β, IL-18, IL-12 and IL-23, were purchased from R&D Systems (Minneapolis, MN). IL-36 cytokines were produced in *E. coli* by R&D Systems. After extraction, refolding and purification of IL-36 cytokines, the level of LPS was determined to be less that 1.0 EU/microgram by the Limulus Amebocyte Lysate method. The molecular weight of recombinant IL-36Ra is 17,000 with an N-terminus at valine 2. On silver stained SDS-PAGE under reducing conditions, there is a single band and the purity is >95%.

PBMC isolation and stimulation | Venous blood from healthy subjects was drawn into 10 mL EDTA tubes after written informed consent was obtained. Blood sampling from healthy volunteers was approved by the ethical board of the Radboud University Nijmegen Medical Centre. Subsequently, PBMCs were isolated as described previously [16]. Blood was diluted in phosphate buffered saline (PBS) (1:1) and fractions were separated by Ficoll (Ficoll-Paque Plus, GE Healthcare, Zeist, The Netherlands) density gradient centrifugation. Cells were washed twice with PBS and resuspended in RPMI-1640 culture medium supplemented with 10 µg/mL gentamicin, 10 mM L-glutamine and 10 mM pyruvate (Gibco, Invitrogen, Breda, The Netherlands). The cells were counted using a particle counter (Beckmann Coulter, Woerden, The Netherlands) and the cell number was adjusted to $5x10^6$/mL. PBMCs were plated in 96-well round-bottom plates (Corning, NY, USA) at a final concentration of $2,5x10^6$/mL and were stimulated with medium, $1x10^7$/mL HI *A. fumigatus* conidia, or live *A. fumigatus* conidia in a total volume of 200 µL. Cells were stimulated for 0 hours, 4 hours, 8 hours, 24 hours and 7 days at 37°C and 5% CO_2. After stimulation culture supernatants were collected and stored at -20°C and the cells were lysed in TRIzol reagent (Invitrogen) and stored at -20°C until RNA isolation was performed. Pattern recognition pathways were inhibited in PBMCs by pre-incubation for 1hour with a specific inhibitor. LPS derived from *Bartonella quitana* was used to block TLR4 [17]. *Bartonella quitana* LPS was extracted and purified as described previously [18]. Mouse anti-humanTLR2 (eBioscience, Halle-Zoersel, Belgium) and control mouse IgG1 (eBioscience) were used at a final concentration of 10 µg/mL. Anti-human integrin β2 (αCR3) and control goat IgG were purchased from R&D systems (Minneapolis, MN) and used in a final concentration of 10 µg/mL. Laminarin (Sigma, St Louis, MO) was used in a final concentration of 50 ng/mL to inhibit dectin-1. Syk inhibitor (3-(1-Methyl-1H-indol-3-yl-methylene)-2-oxo-2,3-dihydro-1H-indole-5-sulfonamide) was purchased from Calbiochem (Merck, Darmstadt, Germany) and was used in a concentration of 50 nM to inhibit Syk signalling. This inhibitor is a cell-permeable oxindole compound that acts as a potent, reversible, and ATP-competitive Syk inhibitor.

IL-36 and IL-36Ra expression | RNA was isolated according to the protocol supplied with the TRIzol reagent. Isolated mRNA (1 µg) was reverse transcribed into cDNA using the iScript cDNA synthesis kit (BIORAD, Hercules, CA). Quantitative real-time PCR (qPCR) was performed using power SYBR Green PCR master mix (Applied Biosystems, Carlsbad, CA) and Primers hIL-1F6-F 5'-TTG-CCT-TAA-TCT-CAT-GCC-GAC-3' and hIL-1F6-R 5'-CCG-ACT-TTA-GCA-CAC-ATC-AGG-3' for amplification of IL-36α, hIL-1F8-F 5'-AGA-AAT-TCA-GGG-CAA-GCC-TAC-3' and hIL-1F8-R 5'-CAG-CCA-GGG-TAA-GAG-ACT-GAC-3' for amplification of IL-36β, hIL-1F9-F 5'-GAA-ACC-CTT-CCT-TTT-CTA-CCG-TG-3' and hIL-1F9-R 5'- GCT-GGT-CTC-TCT-TGG-AGG-AG-3' for amplification of IL-36γ, hIL1F5-FW 5'-ACT-CGG-CAT-TGA-AGG-TGC-TTT-3', hIL1F5-REV 5'-GGG-ACC-ACG-CTG-ATC-TCT-T-3' for amplification of IL-36Ra, humIL-18 F 5'-TGT-CGC-AGG-AAT-AAA-GAT-GGC-T-3' and humIL-18 R 5'-CCT-TGG-TCA-ATG-AAG-AGA-ACT-TGG-T-3' for amplification of IL-18, IL-12p35 hum F 5'-CCT-TGC-ACT-TCT-GAA-GAG-ATT-GA-3' and IL-12p35 hum R 5'-ACA-GGG-CCA-TCA-TAA-AAG-AGG-T-3' for amplification of human IL-12p35 and Beta2M hum F 5'-ATG-AGT-ATG-CCT-GCC-GTG-TG-3' and Beta2M hum R 5'-CCA-AAT-GCG-GCA-TCT-TCA-AAC-3' for amplification of β2M. The PCR was performed using an Applied Biosystems 7300 real-time PCR system using PCR conditions 2 minutes 50°C, 10 minutes 95°C followed by 40 cycles at 95°C for 15 sec and 60°C for 1 minute. The RNA levels of IL-36γ and IL-36Ra were corrected for differences in loading concentration using the signal of housekeeping protein β2M and RNA isolates from freshly isolated PBMCs (T=0) were set at 1.

IL-36 receptor blockade | PBMCs were plated in 96-well round-bottom plates (Corning) at a final concentration of $2.5x10^6$/mL and were pre-incubated for 1 hour with medium or IL-36Ra at increasing concentrations. After pre-stimulation the PBMCs were stimulated for 24 hours or 7 days with medium, $1x10^7$/mL HI *A. fumigatus* conidia or hyphae (7 days) or live *A. fumigatus* conidia (24 hours) at 37°C and 5% CO_2. After stimulation, culture supernatants were collected and stored at -20°C until analysis.

IL-36Ra blockade | PBMCs were plated in 96-well round-bottom plates (Corning) at a final concentration of $2.5x10^6$/mL and stimulated for 7 days with medium, $1x10^7$/mL HI *A. fumigatus* conidia at in the presence of Goat IgG isotype control or Goat anti-human IL-36Ra (R&D Systems) at a concentration of 10 µg/mL. After stimulation, culture supernatants were collected and stored at -20°C until analysis.

RORγ and FoxP3 expression | Supernatant was removed from PBMCs that were stimulated for 7 days with medium or $1x10^7$/mL HI *A. fumigatus* conidia. The cells were re-stimulated for 4-6 hours with PMA (50 ng/mL) (Sigma-Aldrich), ionomycin (1 µg/mL) (Sigma-Aldrich) and Golgiplug (BD Biosciences, Breda, the Netherlands) according to the protocol supplied by the manufacturer. Cells were first stained extracellularly using PeCy7-conjugated anti-CD4 (BD Biosciences). Subsequently the cells were fixed and permeabilized with Cytofix/Cytoperm solution (eBioscience) according to the protocol supplied by the manufacturer. Following permeabilization the cells were intracellularly stained with FITC-conjugated anti-IL-17 (eBioscience) and allophycocyanin-conjugated anti-RORγ (eBioscience), or allophycocyanin-conjugated anti-FoxP3. The RORγ$^+$ IL-17$^+$ CD4$^+$ cells and FoxP3$^+$

cells were detected on a FC500 flowcytometer (Beckman Coulter) and the data were analyzed using CXP analysis software v2.2 (Beckman Coulter).

Cytokine measurement | IL-1β, IL-1Ra, IL-4, IL-6, IL-10, IL-12P70, IL-13, IL-17, IL-22, IL-23, and IFNγ were measured using commercially available ELISAs (R&D Systems or Sanquin, Amsterdam, The Netherlands) according to the protocols supplied by the manufacturer. Detection limits are 39 pg/mL, 780 pg/mL, 78 pg/mL, 15.6 pg/mL, 11.7 pg/mL, 39 pg/mL, 39 pg/mL, 78 pg/mL, 15.6 pg/mL and 7.8 pg/mL respectively.

Receptor expression analysis | Supernatant was removed from PBMCs that were stimulated for 7 days with medium or 1x10^7/mL HI *A. fumigatus* conidia. The cells were stained with PeCy7-conjugated anti-CD4, PE-conjugated anti-IL-12Rβ1, APC-conjugated anti-IL-1R1, and FITC-conjugated anti-IL-18Rα. Cells were detected on a FC500 flowcytometer (Beckman Coulter) and the data were analyzed using CXP analysis software v2.2 (Beckman Coulter).

Statistical analysis | The differences between stimulations with control stimulation versus inhibitors or IL-36Ra were analyzed with the Wilcoxon signed rank test. A p-value of < 0.05 was considered statistically significant (*p<0.05, **p<0.01, and ***p<0.001). All experiments were performed at least twice and data represent results of all experiments performed. Data was analyzed using GraphPad Prism v5.0.

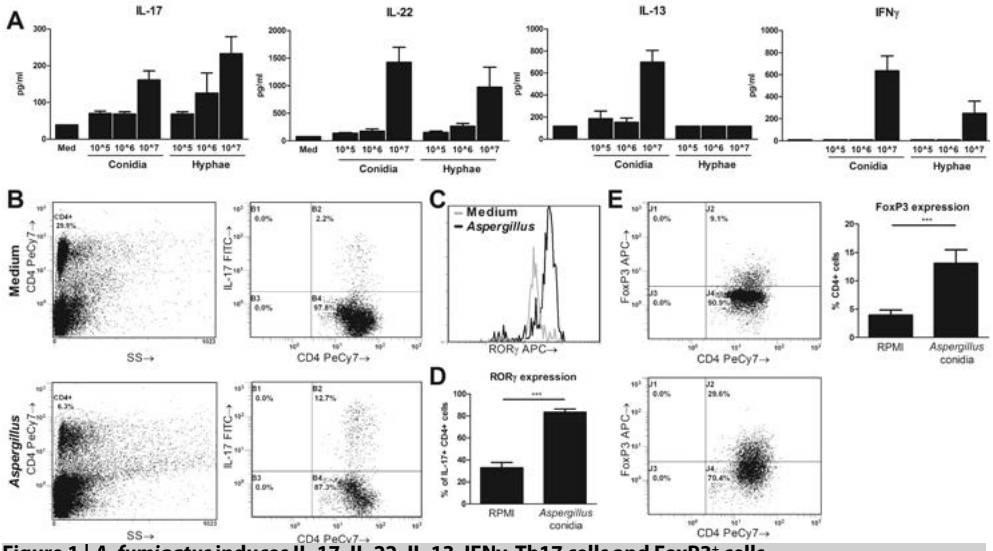

Figure 1 | *A. fumigatus* induces IL-17, IL-22, IL-13, IFNγ, Th17 cells and FoxP3$^+$ cells
(A) IL-17, IL-22, IL-13, and IFNγ concentrations in the culture supernatants of human PBMCs stimulated for 7 days with medium (med), heat-inactivated (HI) *A. fumigatus* conidia or hyphae were determined by ELISA. Data are presented as mean ± SEM of 10 donors. (B-E) After collection of culture supernatants PBMCs stimulated with medium or HI *A. fumigatus* conidia were used for intracellular (B) IL-17 and (C, D) RORγt or (E) FoxP3 staining. Data are shown as mean ± SEM of 7 (D) and 11 (E) donors and are representative of/pooled from 5 (A), 3 (D) and 5 (E) experiments performed. ***p<0.001, Means were compared uring the Wilcoxon signed rank test.

Results

A. fumigatus induces the T-helper cell cytokines IL-17, IL-22, IFNγ and IL-13

To study the effect of IL-36 on pro-inflammatory T-cell responses induced by *A. fumigatus*, we first investigated the capacity of conidia and hyphae to induce in human PBMCs the production of the Th17 cytokines IL-17 and IL-22, and of the Th1 cytokine IFNγ, the Th2 cytokines IL-4 and IL-13 and the anti-inflammatory cytokine IL-10. Increasing numbers of heat-inactivated (HI) conidia were added to PBMC and after 7 days of incubation, IL-17, IL-22, IFNγ, IL-4, IL-13 and IL-10 levels were detemined in the culture supernatants. As shown in Figure 1A, $1x10^7$/mL HI conidia or hyphae induced a IL-17, IL-22, and IFNγ response. IL-13 was induced by $1x10^7$/mL conidia, but not by hyphae (Figure 1A), and production of IL-4 and IL-10 was undetectable after challenge with the fungus (data not shown).

A. fumigatus-induced Th17 cells and FoxP3 cells

We investigated whether *Aspergillus* induced cells with a typical Th17 phenotype ($IL\text{-}17^+$ $ROR\gamma t^+$ $CD4^+$ cells). We gated on $CD4^+$ T cells and subsequently looked at the precence of intracellular IL-17 in PBMCs stimulated with medium or with HI *Aspergillus* conidia, which was increased after stimulation with *Aspergillus* (Figure 1B). Subsequently, we gated on the $IL\text{-}17^+$ $CD4^+$ cells and measured expression of RORγt (Figure 1C). Compared with medium-stimulated PBMCs, *Aspergillus*-stimulated cells demonstrate a significant increase of $ROR\gamma t^+$ $IL\text{-}17^+$ $CD4^+$ cells (Figure 1D). Of the *Aspergillus*-stimulated cells 83.4% (SD ±7.7) $IL\text{-}17^+$ $CD4^+$ cells were $ROR\gamma^+$, compared with non-stimulated cells 32.8% (SD ±13.2). In addition to the $ROR\gamma^+$ Th17 cells, we investigated whether *Aspergillus* induced $FoxP3^+$ cells. Compared with medium only, *Aspergillus* induced a significant expansion of the $FoxP3^+$ population (Figure 1E).

A. fumigatus increases steady state levels of IL-36α, IL-36β, IL-36γ, and IL-36Ra mRNA

We next assessed whether *A. fumigatus* induces the IL-36 receptor ligands and IL-36Ra. The *Aspergillus* cell wall is highly dynamic and therefore will express different pathogen associated microbial patterns (PAMPs) at different stages of infection. Due to a hydrophobic (rodlet) protein layer that covers the resting conidia of *A. fumigatus,* few PAMPs will be exposed [19]. When live conidia are incubated at 37°C in RPMI culture medium they initiate swelling after two to four hours, and form germtubes after six to eight hours (Figure 2A). During the swelling and germination phase, the hydrophobic rodlet layer is lost exposing PAMPs [19]. To investigate the ability of different morphological forms of *A. fumigatus* to induce IL-36 family members, we compared resting conidia which are generaly considered to be poorly immunogenic, with live conidia that are allowed to germinate and expose numerous PAMPs in the process, and hyphae that are exposing numerous PAMPs. Human PBMCs were stimulated the morphological forms for 4 hours, 8 hours, and 24 hours. mRNA of IL-36α, IL-36β, IL-36γ and IL-36Ra by *Aspergillus* was induced by all different morphological forms of *A. fumigatus,* however the magnitute of expression of the IL-36 cytokines differed (Figure 2). IL-36α expression was only significantly induced by HI *Aspergillus* hyphae 4 hours after stimulation, conidia (live and HI) did not

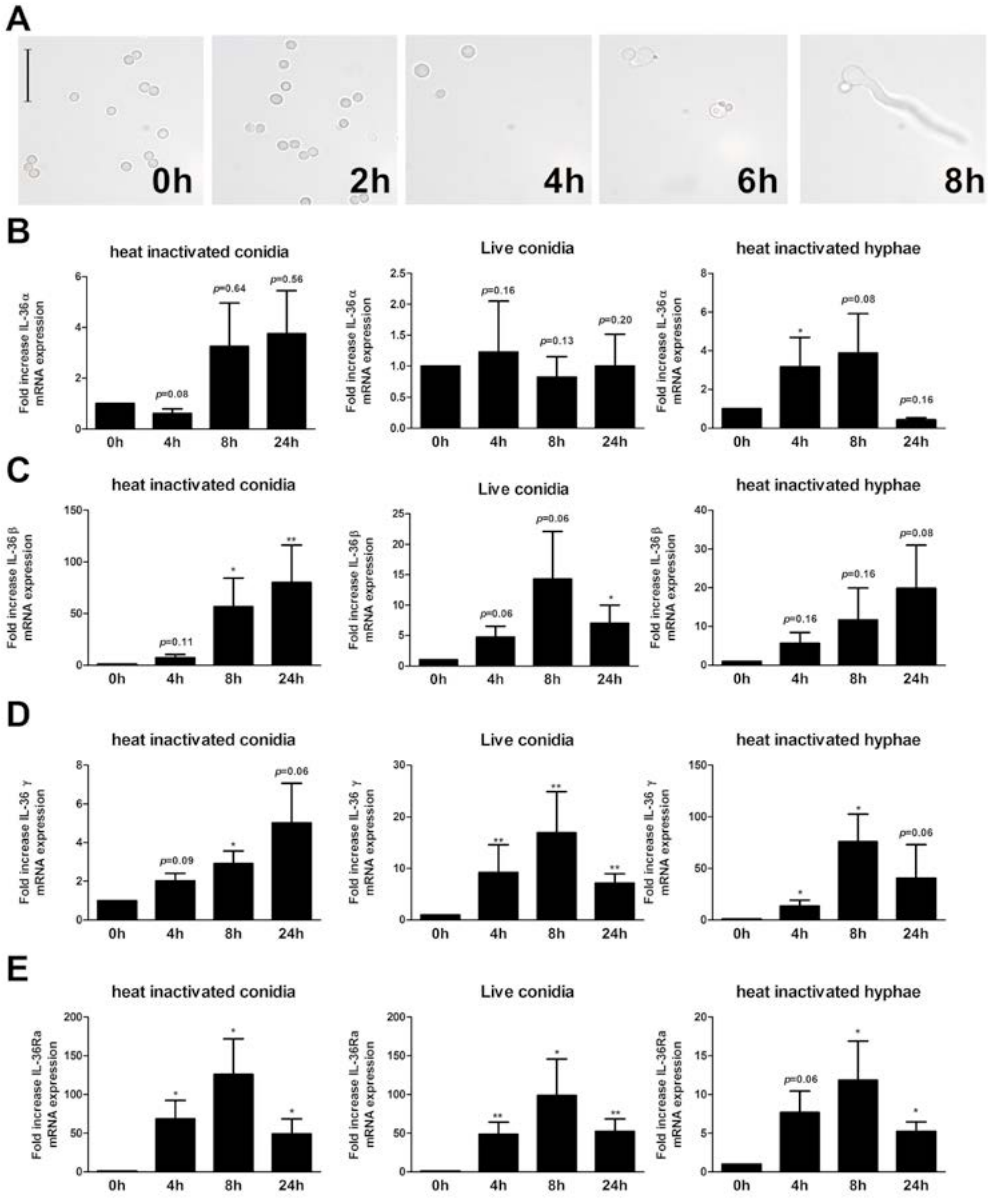

Figure 2 | *A. fumigatus* induces transcription of IL-36α, IL-36β, IL-36γ and IL-36Ra
(A) Microscopic examination of germination of live *A. fumigatus* conidia over a time course of 8 hours at 1000× magnification. The bar represents 10 μm and is representative for each figure since the same magnification was used for all time points. (B) IL-36α, (C) IL-36β, (D) IL-36γ, and (E) IL-36Ra mRNA expression in human PBMCs that were stimulated with HI *A. fumigatus* conidia, live *A. fumigatus* conidia, or HI *A. fumigatus* hyphae was determined by quantitive RT-PCR. Baseline (0 hours) was set at 1 and the fold increase is expressed as mean ± SEM of 6 (HI conidia and hyphae) or 8 (live conidia) donors. Data shown are representative of/pooled from 3 (HI conidia and hyphae) and 4 (live conidia) experiments performed. *$p<0.05$, **$p<0.01$ Means were compared with baseline using the Wilcoxon signed rank test.

significantly induce IL-36α expression (Figure 2B). IL-36β was only significantly induced by heat-killed *A.fumigatus* conidia (Figure 2C). Although stimulation with HI conidia did not result in a significant upregulation of IL-36γ expression, PBMCs stimulated with live conidia or with HI *A. fumigatus* hyphae resulted in a significant induction of IL-36γ transcription (Figure 2D). mRNA of IL-36Ra significantly induced by stimmulationw with all morphological forms of *Aspergillus* and ranged from 15-120 fold increase; dependent on the time of measurement and morphological form of *Aspergillus*. Overall, the expression of mRNA for the IL-36 cytokines was maximal at 8 hours after stimulation (Figure 2).

IL-36Ra inhibits *Aspergillus*-induced IL-17 and IFNγ responses

To elucidate the role of IL-36R in the induction of the *Aspergillus*-specific Th1 and Th17 T-cell responses, we added IL-36Ra to block the IL-36R pathway. Human PBMC were stimulated with *A. fumigatus* conidia and hyphae in the absence or presence of IL-36Ra at increasing concentrations from 0.1 ng to 100 ng/mL. We observed that IL-36Ra significantly inhibited *Aspergillus*-induced IL-17 and IFNγ production, especially in the lower range of the dose response (100 pg/mL to 1 ng/mL) (Figure 3A and 3B). The production of IL-22 was only significantly affected at a concentration of 100 pg/mL IL-36Ra (Figure 3A and 3B). IL-36Ra did not modulate IL-13 (Figure 3A) and proliferation of FoxP3$^+$ cells (Figure 3C) induced by *A. fumigatus* conidia.

Inhibition of endogenous IL-36Ra increases *Aspergillus* induced IL-17 and IL-22

Compared with other fungal pathogens *Aspergillus* is a relatively poor IL-17 inducer. Since, we observed that *A. fumigatus* conidia were potent inducers of IL-36Ra mRNA expression, we wondered whether this relatively low IL-17 induction might be due to high induction of endogenous IL-36Ra in the cultures. To investigate this, PBMCs were stimulated with *A. fumigatus* conidia and endogenous IL-36Ra was inhibited using an anti-human IL-36Ra blocking antibody. *Aspergillus* was able to induce significantly higher IL-17 and IFNγ levels in the stimulations where IL-36Ra was inhibited (Figure 3D). Inhibition of IL-36Ra in medium stimulated cells did not result in significant IL-17 induction.

IL-36 ligands cannot act as surrogates for IL-1β or IL-18

The IL-36R is expressed on CD4 T cells and stimulation with IL-36 ligands results in the induction of IL-17, IFNγ, and IL-4 [5]. In addition IL-1 family members IL-1β and IL-18 can induce IL-17 or IFNγ respectively in the presence of STAT activators, such as IL-12 and IL-23 [20]. To investigate whether IL-36 ligands can directly induce T-helper cytokines we stimulated isolated CD4$^+$ T cells with increasing concentrations of IL-36γ or IL-36β. We did not observe any detectable cytokine concentrations of IL-17 or IFNγ in the supernatants (Figure S1). When IL-36γ or IL-36β was combined with IL-12 or IL-23, we could not detect IL-17 or IFNγ production, in contrast to the combinations of IL-1/IL-23 and IL-18/Il-12 (Figure 4A).

Furthermore, we investigated whether the observed differences in IL-17 and IFNγ production were due to downregulation of the receptors which sense IL-17 and IFNγ inducing cytokines. We stimulated PBMCs with *Aspergillus fumigatus* and subsequently stained the cells for the T-cell marker CD4 and the receptors: IL-1R1, IL-12Rβ1, and IL-18Rα. To determine the presence of the receptors we

gated for the CD4+ cells and dertemined the percentage of positive cells (Figure 4B). There were no significant differences in percentage of CD4+ cells expressing the receptors (Figure 4C) or the mean fluoresence intensity of receptor expression (Figure 4D) between *Aspergillus*-stimulated cells and cells stimulated with *Aspergillus* in the presence of addition of IL-36Ra in the range of 0.1 to 100 ng/mL. There were no differences in the total expression of these receptors in CD4 negative cells, and PBMCs stimulated with medium did not show differences after the addition of IL-36Ra (Figure S2).

IL-36Ra does not affect *Aspergillus*-induced IL-1β, IL-23, IL-18, IL-6, IL-1Ra, and IL-10

We next investigated whether IL-36Ra had an effect on the cytokines that are important for the induction of human Th1 and Th17 responses, such as IL-1β, IL-6, IL-1Ra, IL-23, IL-18 and IL-12p70 production [9]. As a anti-inflammatory cytokine we measured IL-10. Addition of IL-36Ra did not result reduce IL-1β, IL-6, IL-1Ra or IL-10 production induced by *A. fumigatus*. (Figure 4E) IL-12p70 and IL-23 concentrations in the supernatants of PBMCs stimulated with *Aspergillus* were undetectable (data not shown), and no differences were found in mRNA levels of IL-12p35 and IL-18 (Figure S3).

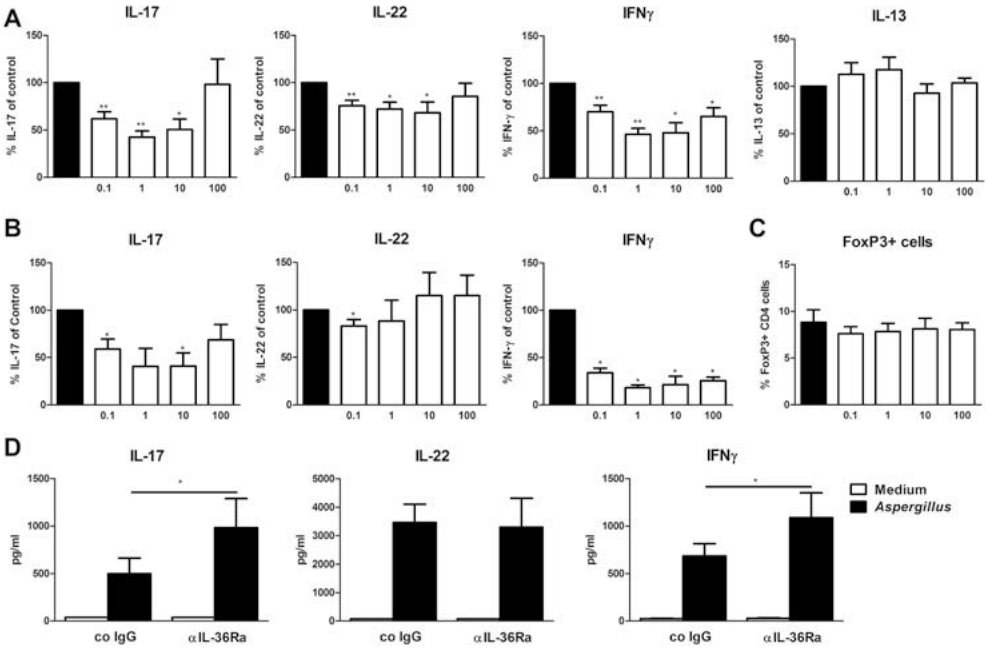

Figure 3 | Inhibition of IL-17 and IFNγ responses by IL-36Ra

IL-17, IL-22, IL-13 and IFNγ concentrations in culture supernatants of human PBMCs that were stimulated for 7 days with HI *A. fumigatus* (A) conidia or (B) hyphae in the presence or absence of IL-36Ra (concentration ranging from 0.1 to 100 ng/mL) were determined by ELISA. The change in IL-17, IL-22, IL-13, and IFNγ is shown as percentage change from control; data are presented as mean ± SEM of 8 (A) or 6 (B) donors. (C) The percentage of FoxP3+ CD4+ cells after stimulation with *A. fumigatus* conidia in presence or absence of IL-36Ra (concentration ranging from 0.1 to 100 ng/mL) was determined by flow cytometry. Data are shown as mean ± SEM of 6 donors. (D) The IL-17, IL-22, and IFNγ concentrations (mean±SEM) in culture supernatants of human PBMCs stimulated with medium or HI *A. fumigatus* conidia in the presence of anti-IL-36Ra or isotype control were determined by ELISA. Data are shown as mean ± SEM of 7 donors. All data shown are representative of/pooled from 4 (A) 3 (B, C, D) experiments performed. *$p<0.05$ **$p<0.01$, Means were compared using the Wilcoxon signed rank test.

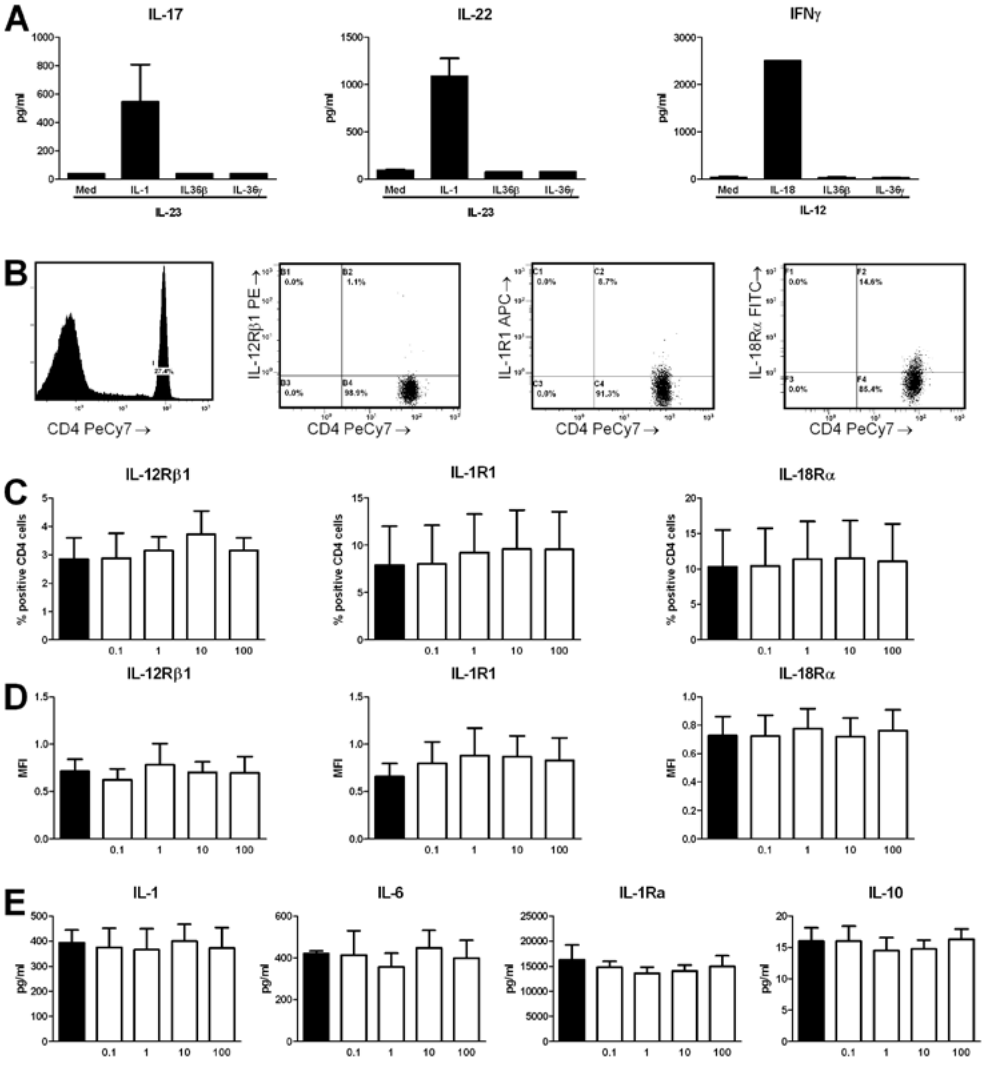

Figure 4 | IL-36 ligands cannot act as surrogates for IL-1β or IL-18

(A) The IL-17, IL-22, and IFNγ concentrations in culture supernatants of PBMCs stimulated with IL-23 or IL-12 alone or in combination with IL-1β (IL-23 only), IL-18 (IL-12 only), IL-36β or IL-36γ were determined by ELISA. (B-D) IL-12Rβ1, IL-1R1, and IL-18Rα expression on $CD4^+$ T cells stimulated for 7 days with *A. fumigatus* in the presence or absence of IL-36Ra (concentration ranging from 0.1 to 100 ng/mL) are shown. Whole PBMCs were stained with anti-CD4, anti-IL-12Rβ1, anti-IL-1R1, and anti-IL-18Rα for flow cytometry. The $CD4^+$ population was gated and (C) the % of positive cells for each of the receptors was determined as well as (D) the mean fluorescence intensity of the signal. mean±SEM. (E) IL-1β, IL-6, IL-1Ra and IL-10 concentrations in culture supernatants of human PBMCs that were stimulated for 24 hours with HI *A. fumigatus* conidia in the presence or absence of IL-36Ra (concentration ranging from 0.1 ng/mL to 100 ng/mL) was determined by ELISA. All data are shown as mean ± SEM of 4 donors and are representative of/pooled from 2 experiments performed.

Induction of IL-36γ by *A. fumigatus* is dependent on dectin-1 Syk signalling and TLR4

The Toll-like receptors (TLRs) TLR2 and TLR4 were previously reported to recognize *A. fumigatus* [21-25]. In addition, the β-glucan receptor dectin-1 and its downstream signalling kinase Syk are also involved in the recognition of *A. fumigatus* [26-29]. To identify whether TLR2, TLR4, CR3, dectin-1 or Syk are required for the induction of IL-36γ by *A. fumigatus*, we stimulated PBMCs in the absence or presence of specific blockers of pattern recognition receptors that have been associated with the recognition of *A. fumigatus*. PBMCs were stimulated with live *A. fumigatus* for 8 hours, since at this time point we observed the highest level of mRNA of IL-36γ (Figure 2B). Inhibition of dectin-1 or Syk resulted in significant reduction of *Aspergillus*-induced IL-36γ transcription (Figure 5A). Blockade of TLR4 also resulted in decreased IL-36γ transcription (Figure 5B). In contrast, when complement receptor 3 (CR3) or TLR2 were blocked, we observed that IL-36γ transcription increased (Figure 5B,C). Taken together these data demonstrate that the dectin-1/Syk and TLR4 pathway are involved in the induction of IL-36γ, whereas TLR2 and CR3 induce signalling that negatively regulates the induction of IL-36γ by *Aspergillus* (Figure 5D).

Discussion

In the present study, we investigated the role of the IL-36R pathway in the induction of *Aspergillus*-specific pro-inflammatory T-helper responses. In addition, we identified the pattern recognition pathways responsible for the induction of IL-36γ by *A. fumigatus*. We demonstrate that *A. fumigatus* induces Th1 and Th17 responses in human PBMCs and that these responses are dependent on the IL-36R pathway. The induction of IL-36γ by *A. fumigatus* is mediated through the dectin-1/Syk and TLR4 pathway, while for unknown reasons, triggering TLR2 and CR3 suppresses the induction of IL-36γ. These data describe a novel cytokine pathway that regulates T-helper responses induced by *A. fumigatus* and describes for the first time the recognition pathways that regulate IL-36 responses in host defence.

Invasive aspergillosis is a devastating disease in the immunocompromised patient and is associated with a high mortality [8]. Adjunctive therapeutic strategies are needed to overcome this significant disease burden in immunocompromised patients. Furthermore, invasive pulmonary aspergillosis is increasingly recognized in non-neutropenic intensive care unit patients that are treated with corticosteroids [30,31]. Knowledge of the host defence against *A. fumigatus* is essential to understand the susceptibility to invasive aspergillosis and to develop new treatment strategies. T-helper responses play an important role in the host defence against *A. fumigatus* [9]. IL-1 family members drive the induction and polarization of T-helper responses [1], and the IL-36 subfamily (IL-36α, β, and γ) induce IFNγ and IL-17 in mouse splenocytes during mitogenic stimulation [5].

Here, we show that the IL-36 receptor pathway plays a role in the *A. fumigatus*-induced Th1 and Th17 responses in human cells, since blockade of the IL-36R with the addition of exogenous IL-36Ra inhibits IL-17 and IFNγ production induced by *A. fumigatus*. Moreover, blocking the endogenous IL-36Ra results in a significant increase in IL-17 and IFN, providing additional evidence that the IL-36R pathway is involved. It is tempting to speculate that the relatively low IL-17 production induced by

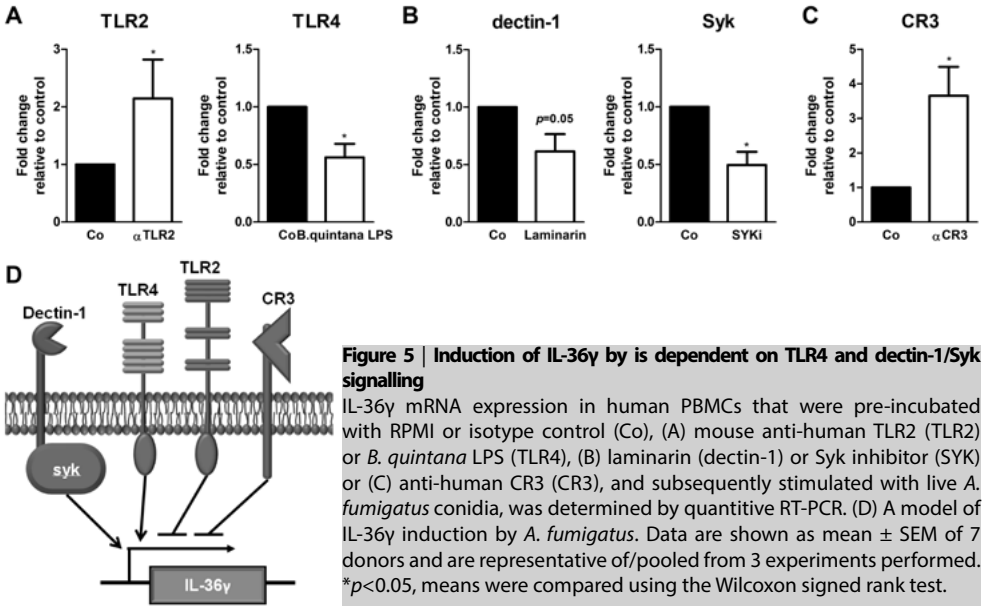

Figure 5 | Induction of IL-36γ by is dependent on TLR4 and dectin-1/Syk signalling
IL-36γ mRNA expression in human PBMCs that were pre-incubated with RPMI or isotype control (Co), (A) mouse anti-human TLR2 (TLR2) or *B. quintana* LPS (TLR4), (B) laminarin (dectin-1) or Syk inhibitor (SYK) or (C) anti-human CR3 (CR3), and subsequently stimulated with live *A. fumigatus* conidia, was determined by quantitive RT-PCR. (D) A model of IL-36γ induction by *A. fumigatus*. Data are shown as mean ± SEM of 7 donors and are representative of/pooled from 3 experiments performed. **p*<0.05, means were compared using the Wilcoxon signed rank test.

Aspergillus compared with other pathogens is due to the capacity of *Aspergillus* to induce IL-36Ra. We observed that low concentrations of IL-36Ra were more efficient in inhibiting T-helper cytokine production than higher concentrations, this effect is still unexplained and needs further investigation. Moreover, we also demonstrate that the different IL-36 family members are induced by different morphological forms of *A. fumigatus*. Interestingly, we observed that heat-inactivated resting conidia, which are generally thought to have a low stimulatory capacity due to their hydrophobic protein layer, are potent inducers of both IL-36 ligands (IL-36β and IL-36γ) and the antagonist IL-36Ra, while hyphae, which are associated with invasive fungal growth, are potent inducers of IL-36γ expression, but induce the antagonist IL-36Ra to a lesser extent. This could indicate that when hyphae are formed during invasive fungal growth, the balance between receptor agonist and antagonist is shifted, however it remains to be determined whether this is detrimental or beneficial for the host.

An important issue that remains to be resolved is whether the effect of IL-36 cytokines on *Aspergillus*-induced T-helper responses is due to an effect of IL-36 cytokines on T cells or dendritic cells (DCs) or both. IL-36 cytokines act directly on murine DCs and murine CD4 T cells. In humans, dendritic cells express high levels of IL-36R and DCs primed with ligands for this receptor are very potent inducers of Th1-cell proliferation [4]. We observed that isolated CD4 T cells stimulated with IL-36 cytokines do not produce IL-17 or IFNγ production. Furthermore, we found that IL-36 cytokines cannot serve as a surrogate for the IL-1 family members IL-1β or IL-18 in inducing Th polarization. Collectively, these data suggest that the primary effects of IL-36 cytokines on T-helper responses is probably through antigen presenting cells, and that IL-36 cytokines might be able to modulate T-helper responses, but only in the setting of activated T-helper cells.

The IL-36R agonist IL-36γ is expressed in the lung and is responsible for neutrophil influx in

a model of house dust mite-induced inflammation in the lung [32]. In human bronchial epithelial cells, IL-36γ induces the chemokines IL-8 and CCL20, which is an important Th17 chemokine [33]. This suggests that IL-36γ can directly recruit neutrophils through the induction of IL-8 and that it is important for CCL20-dependent recruitment of Th17 cells, which in turn will result in additional neutrophil recruitment to the site of infection. Although the TLR3 ligand poly I:C induces secretion of IL-36γ [6,33], other cell surface recetors that induce IL-36R ligands by pathogenic microorganisms have not yet been assessed. We demonstrate that the induction of IL-36γ by *A. fumigatus* is dependent on the TLR4 and dectin-1 pathways, whereas the TLR2 and CR3 pathway reduce the induction of IL-36γ. These pathways are each important for anti-*Aspergillus* host defence [34]. Both TLR4 and dectin-1 polymorphisms have been linked to susceptibility to invasive aspergillosis [35-37], and TLR4 and dectin-1 play a role in the protective anti-*Aspergillus* host defence during a murine model of *A. fumigatus* keratitis [38]. Furthermore, dectin-1 deficiency results in defective IL-17 and IL-22 responses in the lungs of mice challenged intratracheally with *A. fumigatus* [27]. In this model of invasive pulmonary aspergillosis, IL-22 deficient and IL-17 deficient mice show an impaired recruitment of neutrophils in the lungs and increased fungal burden [27]. It was found that monocyte derived macrophages from healthy individuals express IL-1β and IL-1α and later make a switch to IL-36β expression after stimulation with *Aspergillus fumigatus*. While cells of patients with chronic cavitary pulmonary aspergillosis did not show such a switch [39].

Posttranslational processing by the enzyme caspase-1 facilitates the secretion of IL-1 family members IL-1β and IL-18, which play important roles for the induction of protective Th1 and Th17 responses in antifungal host defence [40,41]. Interestingly, IL-36 receptor ligands also require post-translational processing for full agonist or antagonist activity [3]. Although it has been suggested that the release of active IL-36γ from the cell is dependent on caspase-1 [6], the amino-acid sequences surrounding the truncation sites of IL-36γ do not resemble a caspase-1 site [3]. It is therefore possible that other proteases are responsible for posttranslational processing of IL-36γ, and it remains to be elucidated whether the pathways that we identified in the regulation of IL-36γ transcription are involved in the release and activation of IL-36 and IL-36Ra.

The IL-36 receptor pathway has recently gained a lot of interest, since it plays a crucial role in the pathogenesis of psoriasis. Overexpression of IL-36γ in mice results in psoriatic skin lesions and these lesions are exacerbated when IL-36γ was overexpressed in mice that are knock-out for IL-36Ra [42]. Moreover, IL-36 and IL-36Ra are expressed in human psoriatic skin lesions and IL-36Ra polymorphisms lead to familial pustular psoriasis [42-44]. Therefore, the IL-36R pathway represents an attractive therapeutic target for treating psoriasis. Considering this, it is relevant to know the effects of blocking the IL-36R pathway on the host defence against pathogenic microorganisms.

Although further research is required to investigate the role of IL-36 in the immune responses to other pathogens, we have observed that IL-36Ra can also inhibit *Candida albicans*-induced IL-17 and IL-22 production [45]. Furthermore, the IL-36 cytokines do not only regulate the expression and enhance the function of Th17 cytokines, but are also regulated by IL-17 and IL-22 [46]. This suggests a feedback loop between the IL-36 and Th17 cytokines, which makes the IL-36R pathway a central

player in Th17-related mucosal host defence, and the role of this feedback in host defence against fungal pathogens is currently under investigation.

In conclusion, we demonstrate that IL-36R signalling modulates the production of *A. fumigatus*-induced T-helper cytokines IL-17 and IFNγ, and that *A. fumigatus* induces IL-36γ in a TLR4 and dectin-1/Syk-dependent manner. These findings describe for the first time the innate recognition pathways involved in the induction of IL-36R ligands by a microorganism, and describe a novel pathway involved in the induction of T-helper responses by *A. fumigatus*. The future development of therapeutic strategies that will target the IL-36R pathway, and the high mortality associated with invasive aspergillosis, warrants further exploration on the importance of the IL-36R pathway in the host defence against *Aspergillus*.

Acknowledgements

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Supplementary material

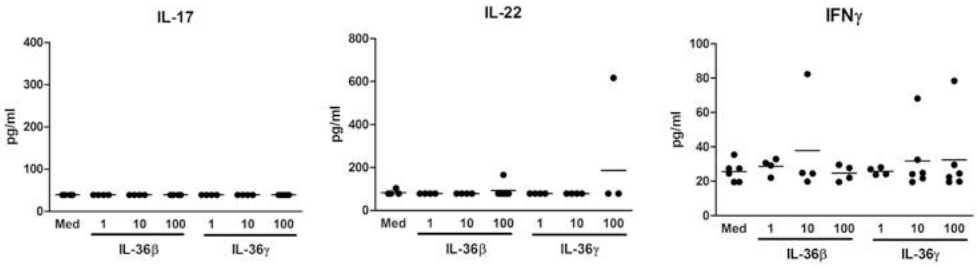

Figure S1 |
IL-17, IL-22, and IFNγ concentrations in culture supernatants of CD4 cells ($5x10^5$) stimulated for 7 days with medium, IL-36β or IL-36γ. Concentrations of IL-36 cytokines range from 1 to 100 ng/mL.

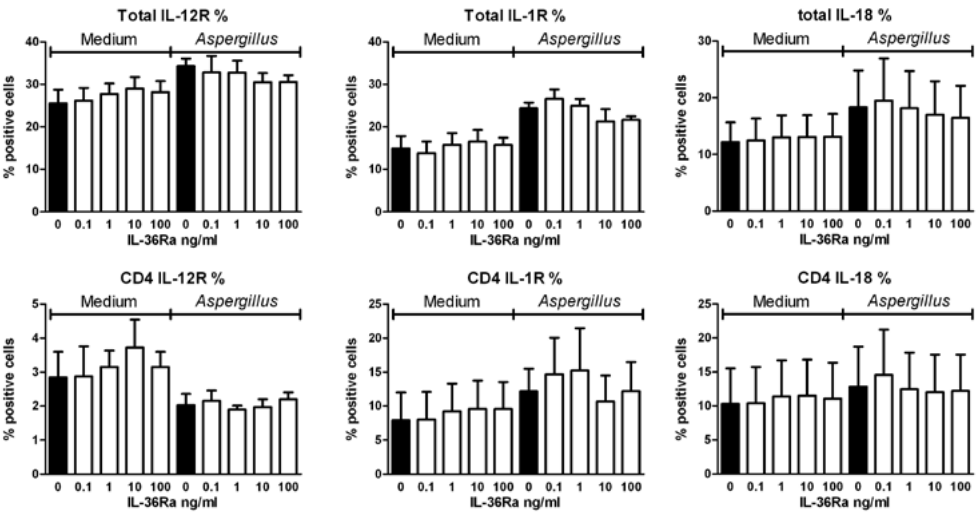

Figure S2 |
IL-12Rβ1, IL-1R1, and IL-18Rα expression on CD4 T-cells and total PBMCs (n=4 donors) stimulated for 7 days with *A. fumigatus* in the presence or absence of IL-36Ra (concentration ranging from 0.1 ng/mL to 100 ng/mL). Whole PBMCs were stained with anti-CD4, anti-IL-12Rβ1, anti-IL-1R1, and anti-IL-18Rα. CD4 positive population was gated and subsequently the % of positive cells for each of the receptors was determined.

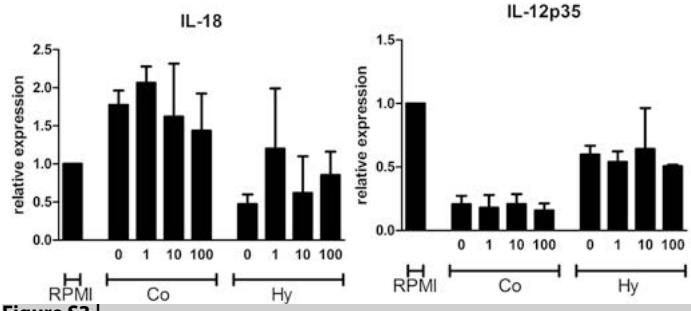

Figure S3 |
IL-18 and IL-12p35 mRNA expression in PBMCs stimulated for 24h with $1x10^7$/mL *Aspergillus* conidia or hyphae in the presence or absence or IL-36Ra in concentrations ranging from 1 to 100 ng/mL.


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Chapter 12

Interferon-gamma as adjunctive immunotherapy for invasive fungal infections: a case series

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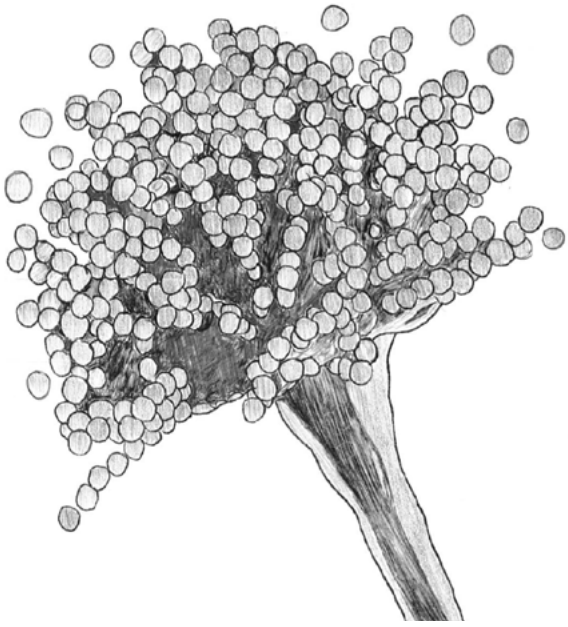

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Abstract

Background | Invasive fungal infections are very severe infections associated with high mortality rates, despite the availability of new classes of antifungal agents. Based on pathophysiological mechanisms and limited pre-clinical and clinical data, adjunctive immune-stimulatory therapy with interferon-gamma (IFNγ) may represent a promising candidate to improve outcome of invasive fungal infections by enhancing host defence mechanisms.

Experimental procedures | In this open-label, prospective case series, we describe eight patients with invasive *Candida* and/or *Aspergillus* infections who were treated with recombinant IFNγ (rIFNγ, 100 μg s.c., thrice a week) for 2 weeks in addition to standard antifungal therapy.

Results | Recombinant IFNγ treatment in patients with invasive *Candida* and/or *Aspergillus* infections partially restored immune function, as characterized by an increased HLA-DR expression in those patients with a baseline expression below 50%, and an enhanced capacity of leukocytes from treated patients to produce pro-inflammatory cytokines involved in antifungal defence.

Conclusions | The present study provides evidence that adjunctive immunotherapy with IFNγ can restore immune function in fungal sepsis patients, warranting future clinical studies to assess its potential clinical benefit.

Trial registration | ClinicalTrials.gov - NCT01270490

Introduction

The incidence of fungal infections is steadily increasing in the last years due to invasive medical diagnosis and immunosuppressive treatment modalities. Despite development of new classes of antifungal agents [1], the invasive fungal infections remain associated with unacceptable high mortality rates and represent a major cause of death worldwide [2-7]. The emergence of significant resistance to the currently available antifungal therapies emphasizes the need for novel approaches to treat invasive fungal infections [8,9]. Invasive fungal infection are most commonly observed in individuals with immune defects or a compromised immune system, and the number of these patients is steadily increasing [10]. Therefore, adjunctive immunotherapy to improve host defence is an attractive strategy to improve the outcome of patients with disseminated fungal infections.

In the past decade, major progress in the understanding of antifungal host responses has enabled the development of a number of novel molecular and cell-based immunotherapeutic approaches for invasive fungal infections [11]. Although invasive candidiasis and aspergillosis are rather different in their pathogenesis, the major protective host response against both fungi is the effective induction of T-helper(Th)1 and IFNγ responses [12-16]. The Th1 cytokine response activates of effector phagocytic cells that kill the fungus [17]. Interestingly, Th1 immunity against *A. fumigatus* was demonstrated to be cross-protective against *C. albicans* [18].

Interferon-gamma (IFNγ), the prototype Th1 cytokine, promotes Th1 differentiation and skews the immune response towards a protective Th1 phenotype [19]. As such, it has been implicated as a treatment option in (invasive) fungal infections [20,21]. Moreover, limited evidence suggests that recombinant IFNγ (rIFNγ) has a beneficial effect on the outcome of fungal infections in patients with chronic granulomatous disease (CGD) [22], HIV [23-25], leukaemia [26,27], and in patients receiving organ transplants [28]. However, it has not been investigated whether rIFNγ actually enhances the immune response in these patients to explain these beneficial clinical effects.

In this report we describe a series of patients with invasive *Candida* and/or *Aspergillus* infections in whom we investigated the effects of treatment with rIFNγ on the host innate and adaptive immune responses.

Experimental procedures

Patients and treatment | To assess the feasibility and preliminary efficacy of IFNγ in combination with anidulafungin for the treatment of candidemia, a single-centre, prospective, randomized open-label pilot (Phase IIIb) study was conducted. This study was registered at ClinicalTrials.gov (NCT01270490) and approved by the local ethics committee of the Radboud University Medical Center. Due to slower than anticipated enrolment rates (from August 2010 until March 2013, only 12 patients could be screened, of which 6 were eligible and provided informed consent [Figure 1]), the study was terminated early. However, during this period, several other patients presented with invasive fungal infections which had an insufficient response to standard antifungal therapy. Although these patients did not meet the inclusion criteria (i.e. presenting with one or more positive

cultures of blood or normally sterile tissue growing *Candida* spp.), they were deemed to benefit from adjunctive immunotherapy as "therapy of last resort" as decided by the attending physician. Within the parameters of standard clinical care these patients were treated according to the same protocol as the patients enrolled in the study, and were therefore included in the present case series. All patients with a history of documented epileptic seizures, pre-existent severe renal impairment (creatinin clearance <30/mL/min) or severe liver failure (defined as a spontaneously increased prothrombin time) were excluded. After obtaining informed consent, eight patients (3 study patients, 5 last resort patients) were treated with rIFNγ (Immukine, Boehringer Ingelheim, 50 μg/m^2 body surface, subcutaneously, three times a week) in addition to standard antifungal therapy as recommended by national and international treatment guidelines [29,30]. Three patients who were included in the Phase IIIb *Candida* pilot-study were assigned to the control group and did not receive rIFNγ.

Blood sampling | Plasma, serum and whole blood specimens were collected at baseline (BL) and serially after the start of antifungal therapy (days 1, 2, 3, 7, 14 and 28). Blood cultures were performed as part of routine care.

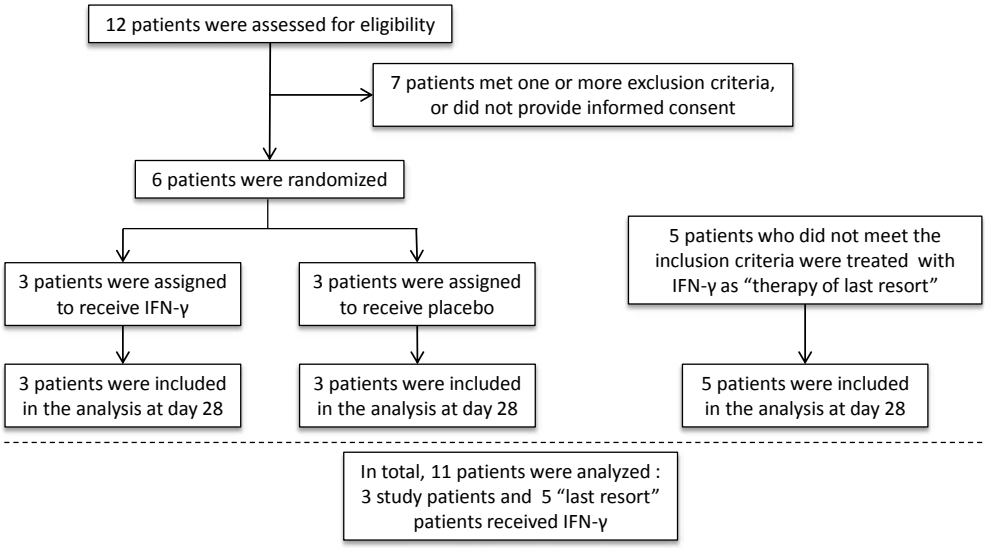

Figure 1 | Screening, Randomization, and Follow-up of the Study Patients
The principal investigator was immediately notified when *Candida spp*. were cultured in blood. With at least one systemic inflammatory response syndrome (SIRS) symptom present in the 24 hours prior to blood culture withdrawal, and administration of systemic antifungal therapy < 72 hours, patients were deemed eligible for the 'IFNγ as an adjunctive treatment for candidemia' pilot-study. In addition, 5 patients not meeting inclusion criteria but who were also treated with rIFNγ as a therapy of last resort, were included in analysis.

Leukocyte populations and surface HLA-DR expression | Heparin anticoagulated blood was stored at 4°C immediately after withdrawal and analysed by flow cytometry. To determine the extent of immune suppression, HLA-DR expression was determined by calculating % HLA-DR-positive cells and HLA-DR mean fluorescence intensity (MFI) within $CD14^+$ cells and various lymphocyte subsets within $CD45^+$ leukocytes$^+$ (see supplementary material for details and a representative flow diagram). Lymphocyte subsets were defined as: T-cells ($CD45^+CD3^+$), T-helper cells (Th, $CD45^+CD3^+CD4^+$), cytotoxic T-cells (Tc, $CD45^+CD3^+CD8^+$), B-cells ($CD45^+CD19^+$), and NK-cells ($CD45^+CD3^-CD56^+$). Subset counts were calculated by multiplying the percentage of gated cells by the total lymphocyte count. Patients with <50% HLA-DR positive monocytes at baseline were considered to exhibit immune paralysis. This threshold of 50% is well below the lower bound of the 99% confidence interval obtained in healthy volunteers in an earlier study of our group using the same methodology in the same laboratory [31]. Therefore mHLA-DR expression levels below 50% are likely to represent immunoparalysis.

Cytokine assays | Venous blood was drawn into 10 mL EDTA tubes, after which peripheral blood mononuclear cells (PBMCs) were isolated as described previously [32]. In short, blood was diluted in phosphate buffered saline (PBS) (1:1) and fractions were separated by Ficoll (Ficoll-Paque Plus, GE healthcare, Zeist, The Netherlands) density gradient centrifugation. Cells were washed twice with PBS and resuspended in RPMI-1640+ (RPMI-1640 Dutch modification supplemented with 10µg/mL gentamicin, 10mM L-glutamine, and 10mM pyruvate) (Gibco, Invitrogen, Breda, The Netherlands). The PBMCs were counted using a particle counter (Beckmann Coulter, Woerden, The Netherlands) and were plated in 96 well round-bottom plates (Corning, NY, USA) at a final concentration of $2{,}5x10^6$/mL, in a total volume of 200 µL. The PBMCs were stimulated for 24 hours, 48 hours, and 7 days with medium alone, or medium containing *E. coli* lipopolysaccharide (LPS; 10 ng/mL), phytohaemaglutinnin (PHA; 10µg/mL), heat-inactivated *Candida albicans* blastoconidia ($1x10^6$/mL) or heat-inactivated *Candida albicans* hyphae (derived from $1x10^6$/mL conidia). After stimulation, cell culture supernatant was collected and stored at -20°C. When all samples were collected, cytokines were measured using commercially available ELISAs (R&D Systems, MN, USA and Sanquin, Amsterdam, The Netherlands) according to the protocols supplied by the manufacturer. Ex-vivo production of cytokines was assessed at timepoints at which their production has been shown to peak [33]. Monocyte derived cytokines such as Interleukin (IL)-1β and tumour necrosis factor (TNF)α were measured in culture supernatants of 24 hour cultures, IL-10 was measured in culture supernatants of 48 hour cultures. T-cell derived cytokines IL-17 and IL-22 were measured in culture supernatants of 7 day cultures.

Statistical analysis | In view of the small sample size, normality of distribution was not assumed. Comparisons of baseline with follow up time points were made using Wilcoxons signed rank test (within-group comparisons, 2 groups). A *p*-value of <0.05 was considered statistically significant. Data are expressed as means ± standard error of the mean. Calculations and statistical analyses were performed using GraphPad Prism v 5.0 (GraphPad Software, San Diego, CA, USA).

Results

Patient characteristics

The patients treated with rIFNγ (5 men, 3 women) had a median age of 49.5 (IQR 28.5-68.8) years. The three female patients in the control group were 36, 51 and 73 years old. Clinical characteristics of the participants are listed in Table 1. Of the 6 patients included in the pilot study, three patients had a positive blood culture for *C. albicans*, two patients for *C. glabrata*, and one patient for *C. tropicalis*. During randomization, the three patients with *C. albicans* cultures were assigned to rIFNγ treatment, whereas the two patients with *C. glabrata* and one with *C. tropicalis* cultures were assigned to the control group. However, no pathophysiological evidence currently exists to suggest that rIFNγ therapy would have a different effect on the immune system in case of *albicans* vs. non-*albicans Candida* infections. Of the other 5 patients treated with rIFNγ as therapy of last resort, three patients had positive cultures for *A. fumigatus* in bronchoalveolar lavage (BAL) fluid, and had proven invasive aspergillosis according to the EORTC/MSG criteria [34]. One patient had a positive blood culture for *C. tropicalis* associated with osteomyelitis. This patient developed new suspected lesions

Table 1 | Summary of clinical characteristics of all patients with invasive fungal infections

IFNγ treated patients						
Age	**Pathogens**	**n=**	**Site of infection**	**n=**	**Antifungal therapy**	**n=**
49.6 ±SD19.8	*C. albicans*	3	Candidaemia	2	Anidulafungin	1
BMI	*Candida spp*	2	Candidaemia Endicarditis	1	Fluconazole	1
22.9 ±SD6.9	*A. fumigatus*	2	Pulmonary aspergilllosis	3	L-AMB + Voriconazole	1
Gender	*C. tropicalis*	1	Osteomeyelitis	1	Voriconazole + Anidulafungin	1
F: 5 M: 3	*A. fumigatus* + *M. genavese*	1	Hepatic abcess	1	Itraconasole, L-AMB, Voriconazole	1
					Anidulafungin and step down to fluconazol	3
Placebo treated patients						
Age	**Pathogens**	**n=**	**Site of infection**	**n=**	**Antifungal therapy**	**n=**
53.0 ±SD19.1	*C. glabrata*	2	Candidaemia	3	Anidulafungin	2
BMI	*C. tropicalis*	1			Anidulafungin + amphotericin B	1
18.5 ±SD4.0						
Gender						
F: 3 M: 0						

on positron emission tomography-computed tomography (PET-CT) while receiving antifungal treatment. In another patient, CT-scan revealed progression of suspected hepatic *Candida* lesions during antifungal treatment. All patients included suffered some degree of immunosuppression: the 6 patients with positive blood cultures for *Candida spp*. had impaired physical barriers due to the presence of indwelling venous catheters (for the need of recurrent blood sampling or total parental nutrition), or an implantable cardioverter-defribillator (ICD) lead. The patient with progression of suspected hepatic *Candida* lesions on the CT-scan, and one patient with multiple pulmonary cavities and *A. fumigatus* in BAL fluid were immunocompromised because of (therapy for) acute myeloid leukaemia. Another patient with acute invasive aspergillosis received immunosuppressive therapy (prednisone and azathioprin) for sarcoidosis and suffered from a co-infection with *Mycobacterium genavense* localised in the bone marrow. A third patient with proven *A. fumigatus* in BAL fluid had a persistent pulmonary cavity after radiotherapy for a T1N0M0 lung carcinoma and developed a chronic aspergillosis. Only two patients, both with acute proven aspergillosis represented by *A. fumigatus* in the BAL fluid, were admitted to the Intensive Care Unit to receive organ supportive therapy (mechanical ventilation and hemodynamic support).

Underlying ilness	**n=**	**Outcome**	**n=**
Stem cell transplantation for AML	1	Cured without further infectious complications	2
Sarcoidosis treated with prednisone and azathioprin	1	Lost to follow up after discharge from hospital	1
First remission induction chemotherapy for AML	1	Slight reduction hepatic lesions	1
ICD, *Streptococcus sanguis* endocarditis, aorta valve replacement with bioprosthesis	1	Cured but complicated with mycotic cerebral aneurysms	1
persistent pulmonary cavity after radiotherapy for a T1N0M0 lungcarcinoma	1	Cured from candidemia episode, 4 months later unrelated bacterial sepsis episode	1
Total parenteral nutrition via Hickmann catheter because of slow transit bowel, intestinal pseudo obstruction, or gastroparesis	3	Died due to infectious complications 71 or 15 days after initiation of IFN-γ therapy	2

Underlying ilness	**n=**	**Outcome**	**n=**
Total parenteral nutrition via Hickmann catheter because of slow transit bowel	1	Cured without further infectious complications	3
HIV with porth-a-cath for venous access	1		
construction of ileal conduit urinary diversion (Bricker deviation) because of pT4N2M1 bladder cancer.	1		

F, female; M, male; ICD, implantable cardioverter-defribillator; HIV, human immunodeficiency virus; L-AMB, liposomal amphotericin B; BAL, bronchoalveolar lavage; AML, acute myeloid leukemia;

Clinical outcome

The three patients in the control group and five out of eight patients treated with rIFNγ recovered uneventfully from the fungal infection (Table 1). Two patients with invasive aspergillosis that were already admitted to the ICU at the time of treatment died due to infectious complications of severe pulmonary aspergillosis, despite rIFNγ treatment. The patient with a *Candida* endocarditis, who despite rIFNγ treatment developed intracerebral mycotic aneurysm, could be discharged from the hospital 93 days after onset of invasive candidiasis.

In all patients treated, rIFNγ was well tolerated. Five patients reported moderate fever upon administration of rIFNγ, which responded well to acetaminophen. Two patients developed liver enzyme abnormalities for which tuberculostatic antibiotics and voriconazol were temporarily discontinued, resulting in recovery of the liver enzyme abnormalities while rIFNγ treatment was continued. No other significant adverse events were observed.

Effect of rIFNγ on ex-vivo IL-1β and TNFα production

To assess the effect of rIFNγ on the capacity of PBMCs to produce pro-inflammatory cytokines, cells were isolated and stimulated before, during, and after treatment. We monitored the fold change in cytokine production compared with baseline (before start of treatment). IL-1β and TNFα are pro-inflammatory cytokines of the innate immune system crucial in the induction and maintenance of the antifungal immune response [35-40]. Before IFNγ treatment, inter-patient variability in cytokine production was high (e.g. TNFα median [IQR] concentration after stimulation with LPS was 792 pg/mL [314-2005]). Nevertheless, in all patients an increase in the capacity to induce different cytokines was observed in the first two days after initiation of IFNγ treatment, independent of their baseline values (group data shown in figure 2), at subsequent time points only a trend towards increased could be observed. In contrast, the placebo-treated patients IL-1β and TNFα responses over time remained similar to baseline. The response against hyphae of *C. albicans* was highly variable between patients. Some rIFNγ-treated patients demonstrated a profound increase of TNFα production after treatment (up to 70 fold), whereas other patients showed no relevant change in TNFα production. Cytokine production remained similar in patients in the control group.

Effect of rIFNγ on ex-vivo IL-17 and IL-22 production

Both IL-17 and IL-22 are cytokines that are thought to be protective in the host defence against invasive fungal infections [35,41-45]. PHA-induced IL-17 and IL-22 production was increased 1 day after initiation of rIFNγ treatment (figure 3). However, at subsequent time points a trend towards increased IL-17 and IL-22 production was observed, which reverted to baseline levels at day 28. Production of IL-17 and IL-22 upon stimulation with *Candida* blastoconidia was elevated after rIFNγ treatment in 6 of 8 patients. Hyphae induced IL-17 and IL-22 production was increased in 4 of 8 and 5 of 8 patients respectively. Patients who received placebo therapy did not display a trend towards increased IL-17 or IL-22 production during the course of treatment (group data shown in figure 3).

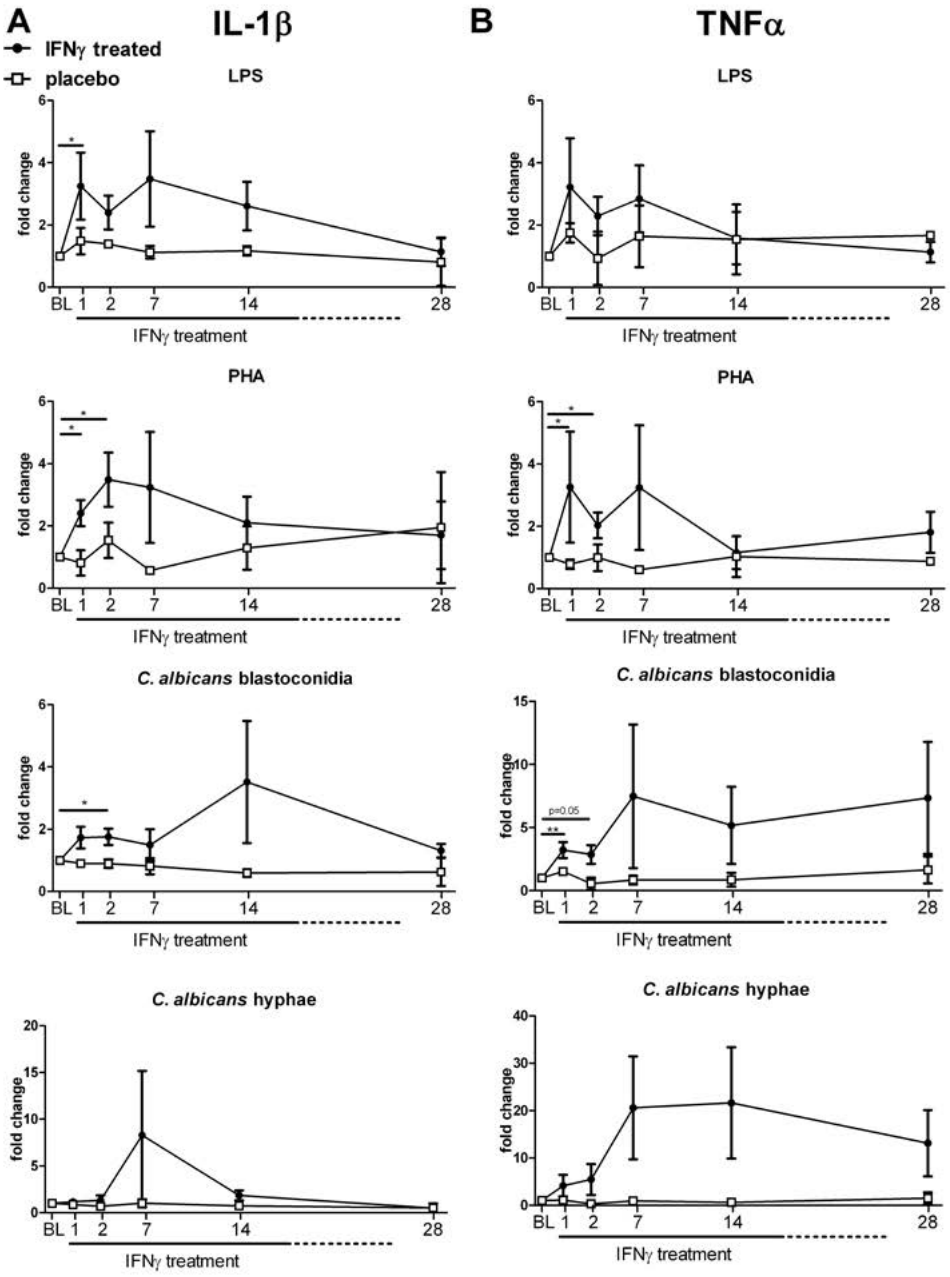

Figure 2 | Effect of rIFNγ on *ex vivo* IL-1β and TNFα production
PBMCs of patients were isolated at baseline and day 1, 2, 7, 14 and 28 after rIFNγ administration. Isolated PBMCs were stimulated for 24 hours with LPS, PHA, *C. albicans* blastoconidia, or *C. albicans* hyphae. (A) IL-1β and (B) TNFα concentrations were measured in culture supernatants. Baseline concentrations were used as control and set at 1; subsequent measurements are plotted as the mean relative fold change ± SEM. Significant change from baseline was determined by subjecting the data to Wilcoxons signed rank test. (*=p<0,05; **=p<0,01).

Effect of rIFNγ on *ex vivo* IL-10 production

In addition to pro-inflammatory cytokines, the capacity to produce anti-inflammatory cytokines can also influence disease outcome. In particular the anti-inflammatory cytokine IL-10 has been associated with protection against immunopathology during severe infections. IL-10 production in response to stimulation with LPS, PHA and *Candida* was highly variable between patients and did not show a distinct pattern following rIFNγ treatment (figure 4). No relevant differences compared to the placebo-treated patients were observed.

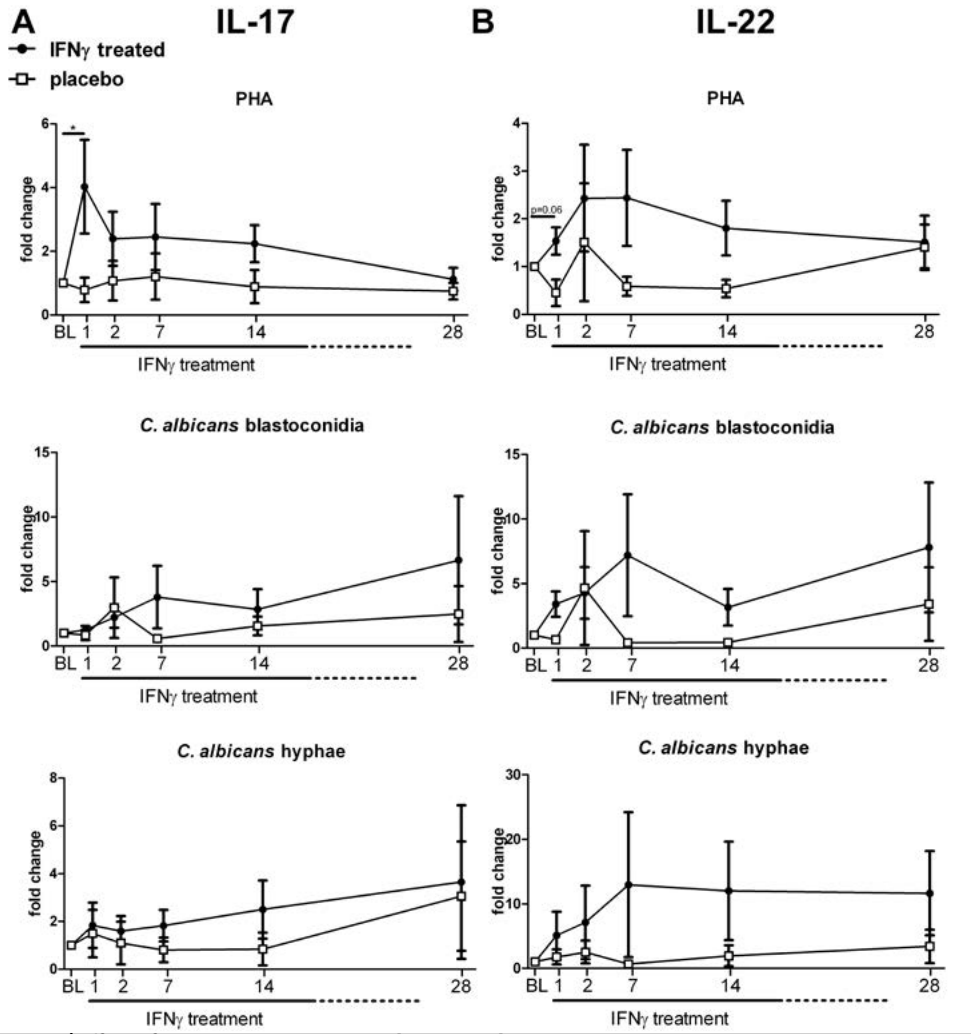

Figure 3 | Effect of rIFNγ on *ex vivo* IL-17 and IL-22 production

PBMCs of patients were isolated at baseline and day 1, 2, 7, 14 and 28 after rIFNγ administration. Isolated PBMCs were stimulated for 7 days with PHA, *C. albicans* blastoconidia, or *C. albicans* hyphae. (A) IL-17 and (B) IL-22 concentrations were measured in culture supernatants. Baseline concentrations were used as control and set at 1; subsequent measurements are plotted as the mean relative fold change ± SEM. Significant change from baseline was determined by subjecting the data to Wilcoxons signed rank test. (*=p<0,05).

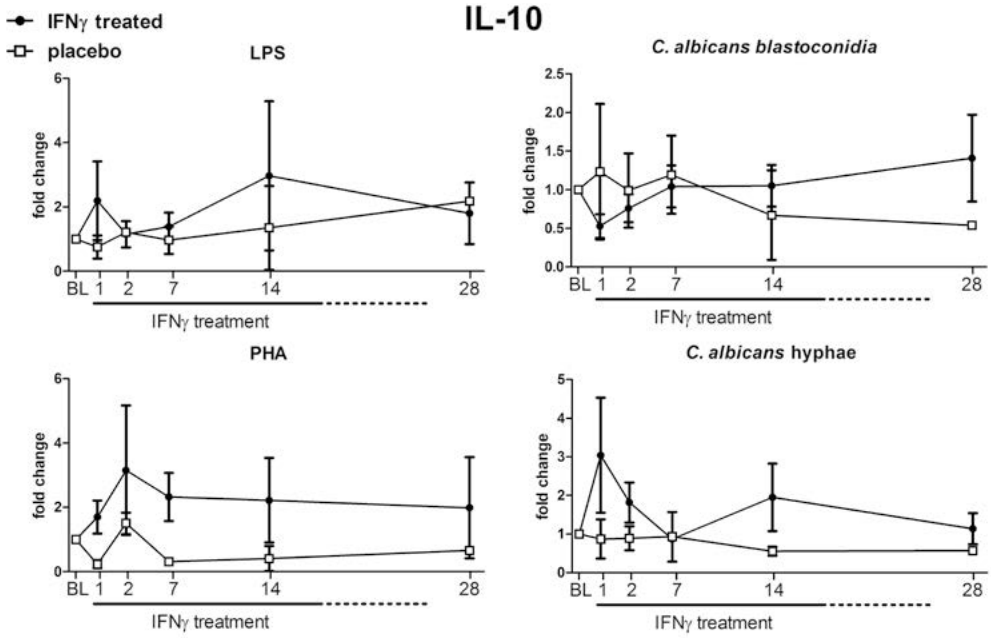

Figure 4 | Effect of rIFNγ on *ex vivo* IL-10 production
PBMCs of patients were isolated at baseline and day 1, 2, 7, 14 and 28 after rIFNγ administration. Isolated PBMCs were stimulated for 48 hours with LPS, PHA, *C. albicans* blastoconidia, or *C. albicans* hyphae. IL-10 concentrations were measured in culture supernatants. Baseline concentrations were used as control and set at 1; subsequent measurements are plotted as the mean relative fold change ± SEM.

HLA-DR expression

The numbers of HLA-DR-positive monocytes, a marker of immunosuppression, varied substantially between patients at baseline (39.05 % [27.5-61.6] vs. 90.6 [88.7-92.5] in IFNγ-treated patients and placebo-treated patients, respectively). Five out of eight IFNγ treated patients exhibited HLA-DR positive monocyte levels below the "immunoparalysis threshold" of 50% and in these patients, an increase of HLA-DR-positive monocytes after IFNγ treatment between 10% and 44% was observed which persisted throughout the study period (Figure 5). Patients with a baseline HLA-DR expression higher than 50% did not show a change in expression. The patient with a HLA-DR-expression <50% who did not show increased levels of HLA-DR positive monocyte numbers at any time point, was one of the two patients who died due to infectious complications. No correlation was found between the level of mHLA-DR expression and TNFα production of LPS-stimulated PBMCs. An inverse correlation of baseline mHLA-DR levels with severity of underlying illness and tissue involvement was found (with higher mHLA-DR levels in patients with only impaired physical barriers, e.g. due to indwelling catheters, compared to patients with impaired immune responses, e.g. due to chemotherapy, immune suppressive agents, bone marrow disease,; data not shown because this compromises patients anonymity).

Cell populations

There were no significant changes in the total leukocyte and granulocyte numbers in rIFNγ-treated patients (Figure S2A). Monocyte counts significantly increased one week after initiation of rIFNγ therapy (Figure S2C) and lymphocyte numbers significantly increased at 2 and 7 days after initiation of rIFNγ therapy (Figure S2D), which could be attributed to slight changes in CD4 lymphocytes (Figure S2E), B-lymphocyte (Figure S2F) and NK-cell numbers (Figure S2G) and a significant increase of CD8 lymphocytes (Figure S2H). No clear changes in leukocyte (subset) counts were observed in placebo-treated patients.

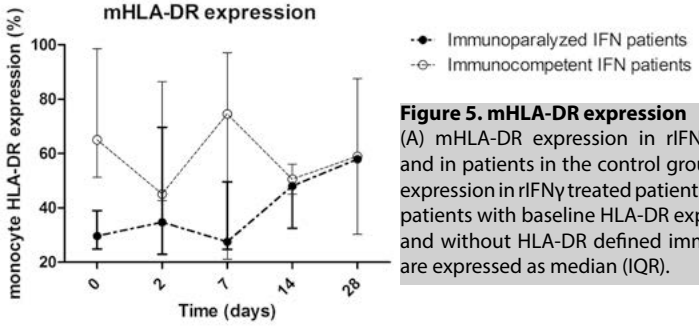

Figure 5. mHLA-DR expression
(A) mHLA-DR expression in rIFNγ treated patients (solid dots) and in patients in the control group (open squares). (B) mHLA-DR expression in rIFNγ treated patients, divided into immunoparalyzed patients with baseline HLA-DR expression below 50 % (solid dots), and without HLA-DR defined immunoparalysis (open dots). Data are expressed as median (IQR).

Discussion

While several small clinical trials illustrated the beneficial clinical effects of adjuvant treatment with IFN-y, the proposed immunostimulating effect of IFNγ as the mechanism of action has not been investigated. In this case series we demonstrate for the first time that adjunctive immunotherapy with rIFNγ improves the leukocyte immune responses in patients with severe invasive fungal infections. This was primarily reflected by increased *ex-vivo* pro-inflammatory cytokine responses of the innate immune system such as IL-1β or TNFα, as well as an increased production of the T-cell cytokines IL-17 and IL-22, which are known to play an important role in the antifungal host defence [35,41-45], and by an increase in HLA-DR expression in mHLA-DR expression in those patients with a low cellular expression as a measure of their immune suppression.

In addition to enhanced *ex-vivo* responses, subtle changes in the leukocyte differentiation were observed following IFNγ treatment. Although there were no significant differences in total leukocyte numbers after treatment with rIFNγ, shifts in leukocyte subpopulations such as increased monocyte and lymphocyte counts were apparent. While lymphocyte numbers increased after rIFNγ therapy, it could not directly be attributed to a specific subset as all of them showed increased values. The most significant increase was that of CD8 cells one week after initiation of rIFNγ therapy. Monocytes and lymphocytes are known to be crucial cells in the host defence against fungal infections. However, the increase of monocytes and lymphocytes during rIFNγ therapy was accompanied by slightly decreased circulating granulocyte numbers. It is not known whether this reduction is due to activation and migration into the infected tissue, or whether a true decrease in granulocyte

generation was induced by the treatment. Although the decrease in granulocyte numbers was slight, the fact that granulocytes, and especially neutrophils, are crucial in the antifungal host defence warrant careful monitoring of granulocyte numbers during IFNγ treatment.

Several clinical studies and case reports have previously demonstrated beneficial effects of rIFNγ in combination with antifungal therapy on outcome of fungal infections (for example in patients with CGD (n=130) [22,46,47], HIV (n=173) [23-25], leukaemia (n=5) [26,27], and transplant patients (n=7) [28], in a patient with *S. aureus* liver abscess and invasive *C. albicans* infection [48], in a patient with intracerebral aspergillosis [49], in two patients with progressive chronic pulmonary aspergillosis [50], and in two patients with idiopathic CD4 lymphopenia and cryptococcal meningitis [51]). However, in contrast to our study, *ex-vivo* immune responses in these patients were not investigated. Due to the limited number of patients and the very heterogeneous population, we could not assess clinical endpoints, although a mean mortality of 25% in the IFNγ treated patients lies below the mean 40% estimated in patients with invasive fungal infections [10,52].

To the best of our knowledge, we are the first to describe mHLA-DR expression, a widely used marker of immunosuppression in (bacterial) sepsis patients [53], in patients with invasive fungal infections. In all IFNγ treated patients who showed baseline mHLA-DR levels below the immunoparalysis threshold of 50% and survived, IFNγ-mediated upregulation of mHLA-DR expression was observed. In agreement with the data presented in this case series, rIFNγ has been shown to significantly increase numbers of HLA-DR-positive monocytes both in a human preclinical bacterial sepsis model and in septic patients [31,54]. Reduced production of TNFα by leukocytes *ex-vivo* stimulated with LPS has also been shown to be marker of immunoparalysis in sepsis patients. In contrast to our study, mHLA-DR expression and *ex-vivo* TNFα production were found to be highly correlated in bacterial sepsis patients [54,55]. A possible explanation for this discrepancy is that, in contrast with the emerging consensus that immunoparalysis renders patients more vulnerable to opportunistic infections in general [53], different defects in immune defences may be responsible for enhanced susceptibility towards different pathogens.

Based on the apparent inverse correlation of baseline mHLA-DR levels with severity of underlying illness and tissue involvement, mHLA-DR levels seem to reflect disease severity and general immune status, and not specific immune defects per se. Hence, patients with invasive fungal infections and associated impaired antifungal immune responses will probably benefit more from immunostimulatory treatment compared to patients with only impaired physical barriers, e.g. due to indwelling catheters and apparent intact antifungal immune responses. Biomarkers reflecting the capacity of specific antifungal immune defences are required to identify patients who suffer from invasive fungal infections due to impaired cell-mediated immunity. It is important to identify such patients and attempt a tailored immunotherapeutic approach guided by the actual level and type of immunoparalysis of that specific patient. A blood based assay has been described that demonstrates a failure to induce IFNγ expression in renal transplant patients and differences in IL-10 and TNFα expression [56], which could be promising biomarkers to identify patients who could benefit from adjunctive immunotherapy.

The intracellular mechanism(s) through which the beneficial effects of IFNγ are mediated remain to be elucidated. Recently it was proposed that IFNγ exerts its effects at the transcription level [57], while others have demonstrated that IFNγ reverses tolerance-associated epigenetic modifications [58]. Another possible mechanism involved in the IFNγ-mediated reversal of immunoparalysis is the downregulation of negative TLR regulators such as IRAK-M, a protein that negatively regulates LPS-induced inflammatory responses and contributes to the development of immunoparalysis [59].

Administration of rIFNγ was tolerated well. Several patients developed a mild fever upon administration, which responded well to acetaminophen treatment. No other side effects were observed. The most important limitation of the present study is the limited number of patients studied. Because the control group consisted of only three patients, no statistical analysis between the treatment and control groups could be performed. However, despite the small sample size, the increase in HLA-DR expression in patients with mHLA-DR expression levels below 50% and the increased *ex-vivo* response of several cytokines that are crucial in antifungal host defence is a promising observation that underlines the potential of immunotherapy. The slow enrolment of patients presenting with candidemia was the main factor contributing to the decision to terminate the phase IIIb *Candida* pilot-study early. With a reported incidence of 2.5-11 per 100.000 persons in Europe [60], and based on previous epidemiological data in our hospital this low enrolment was not expected at the time of the initiation of the study. The much lower incidence of candidemia in the last two years in our hospital is most likely due to a new antibiotic stewardship introduced recently in our hospital, which has reduced the incidence of opportunistic infections. The cut-off value of mHLA-DR expression levels of 50% to distinguish between immunoparalyzed and immunocompetent patients is another limitation of this study, as this is an arbitrary value chosen. We chose this value because it is well below the 99% CI of mHLA-DR values in healthy volunteers [31]. Therefore, patients with mHLA-DR below 50% do have an impaired antigen presenting capacity of their monocytes which we show to be enhanced by IFNγ therapy. Whether this cut-off value truly represents immunoparalysis, reflected by enhanced susceptibility to secondary infections or reduced capacity to clear opportunistic infections, remains to be investigated. Furthermore, the use of a standardized analysis technique to quantify mHLA-DR, such as the Quantibrite method, is preferable, because it facilitates an objective comparison of mHLA-DR expression levels between studies and aids in the definitive establishment of a cut-off value to identify immunoparalyzed patients. Larger studies are required to confirm the data obtained here. To do so, multicentre studies should be facilitated in order to fully explore the potential of IFNγ immunotherapy.

Our data indicate that adjunctive immunotherapy with rIFN-γ in patients with invasive fungal infections partially restores cell-mediated immunity. This suggests that IFNγ treatment enhances antifungal immunity and larger studies are warranted to validate the findings reported here and to assess the impact of IFNγ treatment on clinical outcome. Biomarkers of impaired antifungal immunity should be further investigated in order to identify patients who will benefit most from immunostimulatory therapy.

Acknowledgements

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Supplementary material

Flow cytometric analysis of mHLA-DR expression and lymphocyte subset counts | To ascertain that expression levels did not change due to a delay between withdrawal and analysis, we performed separate experiments on 5 different blood samples. Expression was determined immediately after withdrawal and after 24 hours storage at 4°C. When samples were immediately stored at 4°C after withdrawal and analyzed within 24 hours, we did not observe significant differences in % or MFI compared with samples that were immediately analyzed after withdrawal. Therefore, analysis was performed within 24 hours after immediate storage at 4°C. After withdrawal, 100 µL blood was incubated with the following fluorochrome-conjugated monoclonal antibodies, for 15 minutes protected from light at 4°C. After erythrocyte lysis (NH_4CL: 180 mL + 20 mL lysis stock dilution [BD Pharm-Lyse, BectonDickinson]), cells were washed three times in PBS and monocytes and lymphocytes were identified in a 8-color immunophenotyping (NAVIOS flow cytometer, Beckman Coulter, Miami). Monocytes and lymphocytes were identified by forward and side scatter and by cell-specific binding. The following monoclonal antibodies were used for monocyte HLA-DR analysis: HLA-DR-PE (Immu-357), CD14-ECD (RMO52), CD45-KO (J33). Lymphocyte subpopulations were identified by gating on the lymphocyte population in the CD45/SS plot followed by a gating on CD3-APC (UCHT1), CD4-PECy5.5 (13B8.2), CD8-APCAlexa700 (B9.11), CD19-APCAlexa750 (HD37) and CD56-PECy7 (N901) to determine the helper T cells, cytotoxic T cells, B cells and NK cells within the lymphocyte gate (all MoAbs were obtained from Beckman Coulter, Marseille, France).

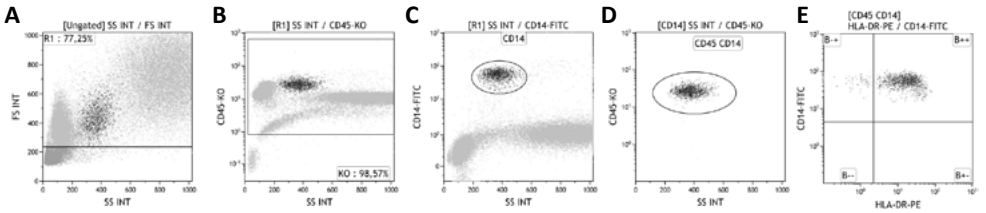


Figure S1 | Representative flow diagram of monocyte HLA-DR measurements
(A) Heparin blood was first analysed on forward- and side scatter to exclude cell debris and erythrocytes. (B) Subsequently, $CD45^+$ cells were selected and (C) within the $CD45^+$ fraction was gated for $CD14^+$ cells. (D) The $CD45^+$ $CD14^+$ cells (E) were analysed for the percentage of HLA-DR positivity.

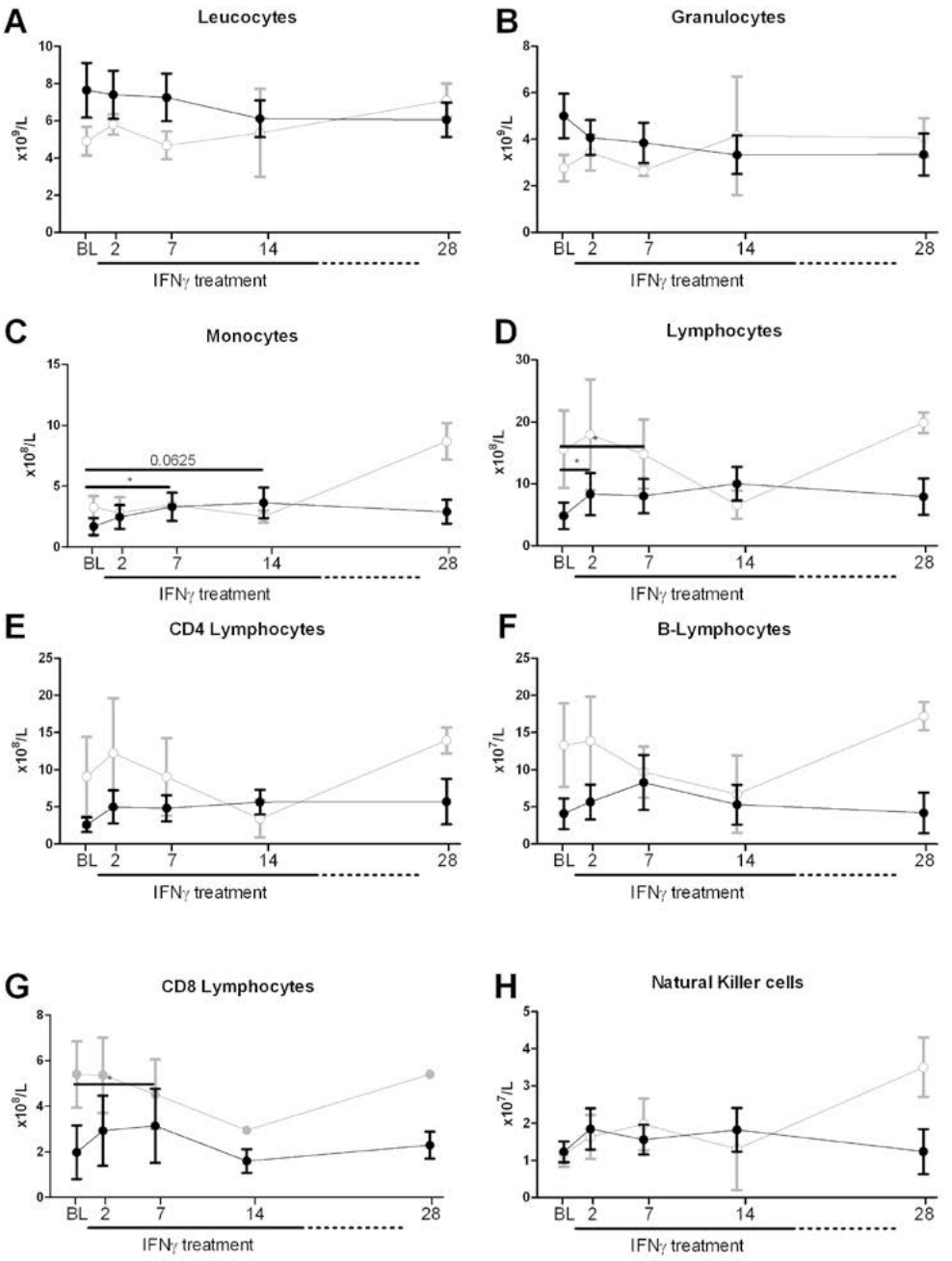
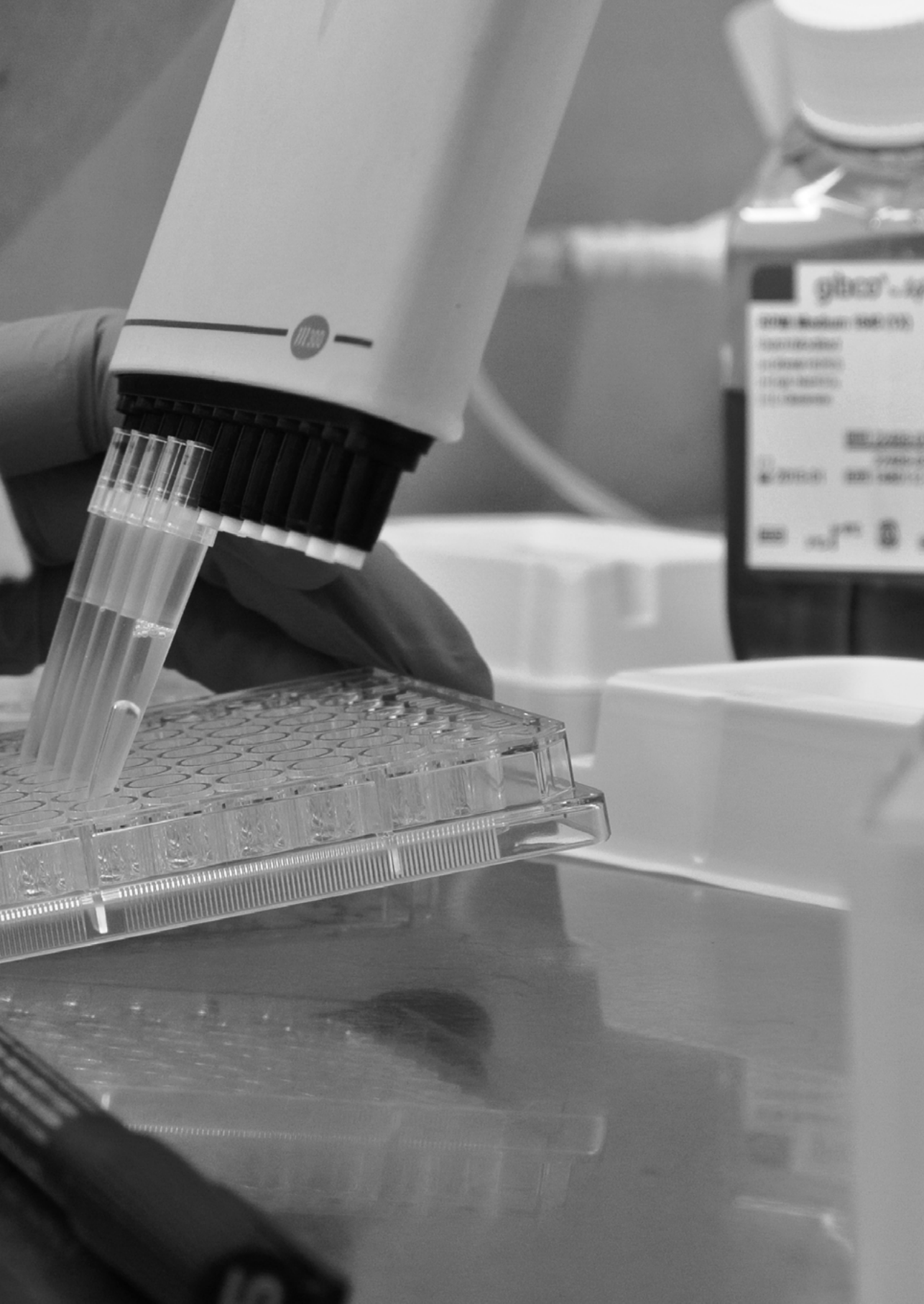
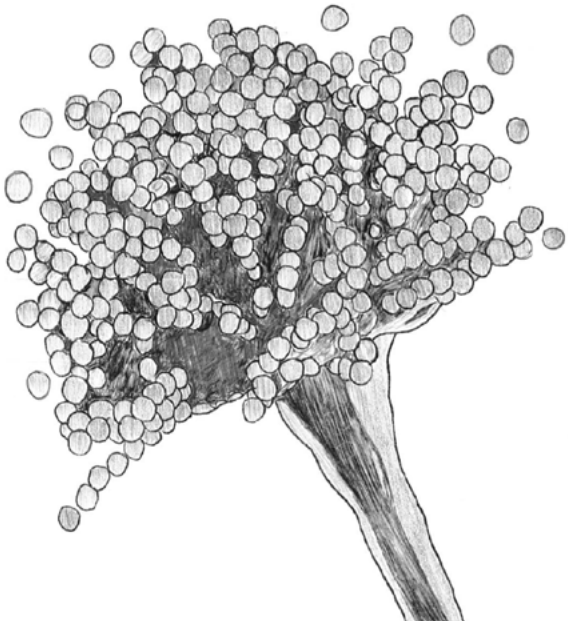


Figure S2 | Changes in immune cell populations
(A) Total leukocyte numbers and (B) numbers of granulocytes, (C) monocytes and (D) lymphocytes measured in peripheral blood. (E) Numbers of CD4 lymphocytes, (F) B-lymphocytes, (G) CD8 lymphocytes and (H) NK cells within the lymphocyte population were quantified using flowcytytometry.



Chapter 13

General discussion and future perspectives



General discussion

The research within this thesis aimed to explore the recognition and cytokine signalling pathways that play a role in the host defence against *Aspergillus fumigatus.* Here we studied the pattern recognition receptors (PRRs) involved in the host defence against *A. fumigatus* and elucidated that the dectin-1/ spleen tyrosine kinase (Syk) pathway is an important regulator of LC3-associated phagocytosis. Furthermore, we dissected which PRRs were involved in the induction of the T-helper(Th)1, Th17, Th22 and Th2 subsets in response to *A. fumigatus*. We found that a deficiency in the intracellular receptor NOD1 protects immunocompromised mice against invasive aspergillosis. In the second part of the thesis our studies explored cytokine signalling in the host defence against *A. fumigatus.* We discussed the relevance of the interleukin(IL)-1 pathway in aspergillosis and were able to show that common genetic variants in IL-1 family genes predispose solid organ transplant patients for invasive aspergillosis. We demonstrated that the molecule galactosaminogalactan of the *Aspergillus* cell wall selectively interferes with IL-1 signalling. In addition, we discovered that the novel IL-1 family cytokines, the IL-36 cytokines, play an important role in regulating *Aspergillus*-induced Th1 and Th17 responses. Furthermore, we explored the potential of adjunctive immunotherapy with recombinant IFNγ in a case series of patients with invasive fungal infections. Collectively, these studies contribute to the rapid developing research field of antifungal host defence mechanisms against *A. fumigatus.* Knowledge from these host defence mechanisms are important in order to be able to identify and design novel treatment strategies.

Several key findings in this thesis have the potential to have an impact on the treatment of invasive aspergillosis, and they need to be explored in more detail:

- **How crucial is LC3-associated phagocytosis in the host defence against *A. fumigatus*?**
- **Is NOD1 a potential target for immunotherapy?**
- **What is the importance of the IL-1 pathway in aspergillosis?**
- **What should be the aim of immunotherapy for aspergillosis?**
  to modulate or to stimulate the host response?

How crucial is LC3-associated phagocytosis in the host defence against *A. fumigatus?*

Autophagy is an ancient mechanism where the cell "eats itself" to remove unwanted or non-required cellular components [1]. It is considered to be a primary response to stressful situations where recycling of cellular components can be used as a source for energy and trace molecules. However, autophagy proteins can also localise to a phagosome when Toll-like receptors are activated [2]. This non-canonical form of autophagy has recently been termed as LC3-associated phagocytosis (LAP). Interestingly, recruitment of autophagy proteins to the phagosome is crucial for killing phagocytosed pathogens [3] and plays an important role in regulating antigen presentation [4].

We investigated in **chapter 3**, the induction of LAP by *A. fumigatus*, and found that the β-glucan activating the dectin-1/Syk pathway plays a major role in inducing LAP. For years dectin-1 has been associated as a crucial molecule for an adequate host defence against *A. fumigatus* [5,6], and was found to promote MHCII antigen presentation through LAP [7]. In our studies we not only confirmed the role of dectin-1 and Syk in inducing LAP, but we were able to identify that corticosteroids interfere with LC3-associated phagocytosis of *Aspergillus*. Corticosteroid treatment has been associated as an important risk factor for aspergillosis [8], due to blocking tyrosine kinase phosphorylation in T- and B-cells [9-11], and block reactive oxygen species (ROS) production in macrophages during fungal infection [12]. It is tempting to speculate that the interference of corticosteroids with LAP is another mechanism why corticosteroids contribute to this increased risk of developing invasive aspergillosis. Moreover, patients with chronic granulomatous disease are incapable of generating NADPH dependent ROS and are uniquely susceptible to *Aspergillus*. In **chapter 3** we identified that CGD patients are deficient in LAP, which was supported by another study [13]. Furthermore, we were able to show that anakinra (blocking the IL-1 receptor) was able to restore LAP in CGD mice which protected them from aspergillosis [13]. This suggests that the defect in LAP in CGD contributes to their high susceptibility to invasive mould infection and that the IL-1 pathway could have an important role in controlling LAP.

Since corticosteroids inhibit crucial host defence mechanisms against *A. fumigatus,* one could argue that treatment of immunopathology associated with chronic pulmonary aspergillosis or allergic pulmonary aspergillosis (ABPA) with corticosteroids might not be the best treatment option, and that more targeted treatment strategies are needed. Nevertheless, corticosteroids are widely used for severe exacerbations of ABPA. In **chapter 5** we demonstrate that treatment of cells isolated from ABPA patients with IFNγ can reverse the Th2 bias that is associated with the pathogenesis of ABPA. Clinical studies are therefore required to investigate whether treatment with IFNγ could be used to reduce or even replace corticosteroid therapy in this patient group.

Is NOD1 a potential target for immunotherapy?

NOD receptors recognise bacterial peptidoglycan [14,15], but recent reports suggest that NOD receptors could be involved in the recognition of *Aspergillus* as well [16-19], despite the fact that *Aspergillus* does not express peptidoglycans. In our studies in **chapter 6** we found that NOD1 deficient mice were protected against aspergillosis, despite receiving cyclophosphamide-induced immunosuppression that results in wild-type mice being extremely susceptible to invasive aspergillosis [20]. Only one report

has shown an interaction between *Aspergillus* and the NOD1 receptor in corneal epithelial cells [19], but we could not have anticipated that NOD1 deficiency could be protective and enhance fungal killing. To our knowledge this is the first report that demonstrates that the absence of NOD1 signalling is beneficial for survival and killing of a (opportunistic)pathogen, as NOD1 was previously demonstrated to be required for killing of *Helicobacter pylori* [21] and plays an important role in the pulmonary host defence against *Mycobacterium tuberculosis* [22] and *Pseurdomonas aeruginosa* [23]. We observed that the absence of NOD1, but not NOD2, improved fungal killing and resulted in increased release of reactive oxygen species. These results could be very important, especially when bearing in mind that ROS production is crucial for protection against invasive aspergillosis [24]. In addition, we found that NOD2 deficient mice had an increased survival, but demonstrated severe symptoms of illness. Moreover, cytokine responses were found to be decreased in NOD2 deficient cells of both humans and animals, while ROS production and fungal killing was not enhanced in NOD2 deficient cells. Follow up studies are need to dissect the protective effect of NOD1 and comparing it with the effects of NOD2 deficiency. Moreover, both NOD receptors share the same downstream signalling kinase RICK, and it is therefore surprising that deficiency of either receptor results in a different phenotype. Such differential regulation by the NOD receptors has been previously observed in murine arthritis model [25]. We therefore suggest that our current data warrants further investigation of the NOD1 receptor as a potential target for immunotherapy of invasive aspergillosis in immunocompromised patients.

What is the importance of the IL-1 pathway in aspergillosis?

Cytokines of the interleukin-1 (IL-1) family play an essential role in the host defence during infection, but are also associated with detrimental acute and chronic inflammation. The IL-1 family consists of 11 members and the most well studied cytokines of the IL-1 subfamily are: IL-1α, IL-1β and IL-1Receptor antagonist (Ra)[26]. To illustrate the importance of this IL-1 subfamily, we demonstrated in **chapter 9** that common genetic variants in *IL1B* and *IL1RN* are associated with invasive fungal infection in solid organ transplant patients. This is in line with previous studies that demonstrated that polymorphisms in IL-1 genes can predict the risk for invasive aspergillosis following hematopoietic stem cell transplantion [27], and that IL-1 polymorphisms were associated with chronic cavitary pulmonary aspergillosis [28]. Interleukin-1 plays a crucial role in early neutrophil recruitment via direct effects [29,30], but also regulates recruitment of neutrophils during later stages of infection through induction of the T-helper 17 subset [31]. We were able to identify In **chapter 4** that IL-1 signalling is crucial for the induction of the Th17 cytokines IL-17 and IL-22 by *Aspergillus*, however we found IL-1 also to be crucial for induction of the Th1 cytokine IFNγ. In **chapter 5** we demonstrated that IL-1 neutralization abolishes *Aspergillus*-induced Th2 responses. These data suggest that IL-1 is a general regulator of T-helper subsets and not only the Th17 subset, as was described earlier. Furthermore, we identified in **chapter 8** that galactosaminogalactan from the cell wall of *A. fumigatus* can interfere with IL-1 signalling by specifically inducing IL-1Ra thereby reducing Th17 responses and making mice more susceptible to aspergillosis. An attractive strategy would be to target this molecule in order to relieve the inhibition of the IL-1 pathway. However, in allergic aspergillosis or

chronic aspergillosis immunopathology driven by IL-1, and IL-1 itself could be a potential target for immunotherapy. Moreover, the detrimental hyperinflammatory response in CGD is driven by IL-1 [32]. Despite the fact that ROS was found to get *Aspergillus*-induced inflammasome activation [33], in CGD patients ROS independent activation of the inflammasome was observed [34,35]. In these patients blocking of IL-1 reduced this detrimental inflammatory response resulting in increased survival of CGD mice with aspergillosis [13]. Thus IL-1 plays a prominent during aspergillosis, however whether IL-1 signalling is beneficial or detrimental for the outcome of aspergillosis depends on the timing, type of infection, and the underlying defects that result in aspergillosis.

In addition to the IL-1 subfamily, we found in **chapter 11** that the novel IL-1 family members IL-36α, β, γ and IL-36Ra regulate the induction of T-helper responses by *Aspergillus*. IL-36 cytokines are primarily associated with skin disorders such as psoriasis [36-39] and reports suggest that these cytokines can play an important role in the pulmonary host defence by modulating pulmonary neutrophil recruitment [40]. These observations suggest that IL-36 cytokines could play a role during pulmonary aspergillosis, but future studies are required to confirm a role for IL-36 *in vivo*. However, the description of a novel cytokine pathway that regulates Th1 and Th17 responses against *Aspergillus* opens up new strategies for potential targets for immunotherapy and warrants future studies to investigate whether common genetic variations in the IL-36 genes also influence susceptibility against aspergillosis.

Furthermore, another cytokine of the IL-1 family that could potentially be of importance in the host defence against *Aspergillus fumigatus* is IL-37. IL-37 is a potent anti-innflammatory cytokine that suppresses the immune response through IL-1R8 (SIGIRR) and subsequently SMAD3 [41]. Of specific interest, this cytokine was found to diminish airway inflammation in mice [42]. Both IL-1R8 [43,44] as well as IL-37 [43] itself have been found to play a role in the host response against *A. fumigatus* by suppressing immunopathology. However, in the host defence against the opportunistic pathogen *Candida albicans* IL-37 was found to decrease the capacity to recruit neutrophils and mount cytokine responses [45], antifungal host responses that are also important for the host defence against *A. fumigatus*. Therefore, in an experimental model for *Aspergillus* where immunopathology plays a less significant role IL-37 could potentially also be detrimental by impairing innate antifungal host defence mechanisms.

Collectively, a prominent role of IL-1 cytokine family in the host defence against *A. fumigatus* is illustrated. However, this is a role with two sides: pro-inflammatory immune responses are required for an efficient clearance of *Aspergillus* conidia, yet activation of pro-inflammatory immune responses could also lead to massive neutrophil recruitment which is associated with detrimental immunopathology. Still, since the cytokines of the IL-1 family seem to play a key role in host defence against *Aspergillus* they should be considered potentially promising targets for immunotherapy. In addition, polymorphisms in the IL-1 family could be potential markers for susceptibility screening.

What should be the aim of immunotherapy in aspergillosis:
to modulate or to stimulate the host response?

The treatment of aspergillosis poses a serious challenge for clinicians worldwide. Patients often have a complicated immune status that predisposes them to develop invasive, chronic or allergic

aspergillosis. Furthermore, resistance to antimycotic therapy is being increasingly reported [46,47], even the use of agricultural azoles is being associated with antimycotic resistance of clinical isolates [48,49]. Patients that suffer from aspergillosis rarely have a normally functioning immune response, they are either immunosuppressed, have non-resolving inflammation or mount hyperinflammatory responses against the fungus. Therefore, modulation of the host response in these patients could be an attractive adjunctive treatment strategy.

In **chapter 12** we investigated whether adjunctive treatment with IFNγ benefits patients with invasive fungal infections, and found that IFNγ boosted the patient's ability to mount a cytokine response against *C. albicans*. However, patients that received hematopoietic stem cell transplantation go through a period where the immune system is absent, and are not likely to benefit from immunostimulatory therapy. In such patients infusion of *ex vivo* differentiated *Aspergillus*-specific T-cells is being suggested as a potentially promising immunotherapy [50], and studies have already demonstrated that the generation of "clinical grade" *Aspergillus*-specific T-cells is possible [51] and that infusion of such T-cells can help to prevent *Aspergillus* infections in HSCT patients [52].However, it is questionable whether such immunostimulatory therapy is beneficial for all patients. Inflammatory responses during aspergillosis has two sides, one side where the host response is required to clear the infection and another side where the host response is causing collateral damage to the host. The hyperinflammatory Th2 response plays a distinct role in the pathogenesis of ABPA [53] and excessive pro-inflammatory responses contribute to the pathology during CPA [54]. Therefore, random immunostimulatory therapy could potentially worsen outcome of infection in these patient groups. In **chapter 5** we investigated several biologicals that could suppress detrimental Th2 responses. Blockade of IL-1 or TNF suppressed Th2 responses. However, protective Th1 responses that are important for an effective clearance of *Aspergillus* were also suppressed [55-57]. We therefore suggest that treatment with IFNγ could be beneficial by suppressing detrimental inflammation driven by Th2 and restoring protective host defence by supplementing the decreased IFNγ production observed in these patients. However, alternative approaches that act on the Th2 axis are being investigated, Vitamin D for example was found to attenuate Th2 responses in CF and ABPA patients [58]. The targeting of regulatory T-cells in ABPA might be another potential immunotherapy due to their capacity to suppress potentially detrimental Th2 and Th17 responses in the lung [59]. This hypothesis is further supported by studies that have demonstrated that regulatory T-cells can attenuate asthma [60,61], and that regulatory T-cells are found to play an important role in preventing detrimental immunopathology in aspergillosis [62-64].

Due to the various options for immunotherapy, screening the patient's host response prior to therapy could be a possible strategy to decide which immunomodulatory approach might be useful as adjunctive therapy to antifungals. For example, a patient that fails to mount a Th1 response against *Aspergillus in vitro* might benefit from recombinant IFNγ therapy. However, when a patient is already capable of mounting a Th1 response, he/she might benefit more from an alternative approach. Because, the clinical presentation of aspergillosis varies among patients, and patients have a wide variety of co-morbidities it is important to stratify patients according to their immune status in order to design personalized treatment strategies for adjunctive immunotherapy.

Future perspectives

Although we tried to provide in these studies additional insight to our understanding of the interaction between the host response and *A. fumigatus*, our current knowledge of the host-*Aspergillus* interaction is far from complete and still many challenges remain that require future attention. For example, which PAMPs of *A. fumigatus* trigger the pattern recognition receptors CR3, TLR2, TLR4, NOD1, and NOD2? In addition, what are the receptors that recognize *Aspergillus* cell wall components such as chitin and galactosaminogalactan? For immunotherapeutic strategies it still remains difficult to predict whether promoting pro-inflammatory immune responses is needed in order to more efficiently clear the fungus, or suppression is needed to prevent detrimental inflammatory pathology during the infection. Continuing our efforts to understand the complexity of the immune system and its interaction with *A. fumigatus* will help us to answer these questions and further elucidate novel cytokine and recognition pathways that are of importance for developing novel treatments and (genetic) susceptibility screening.

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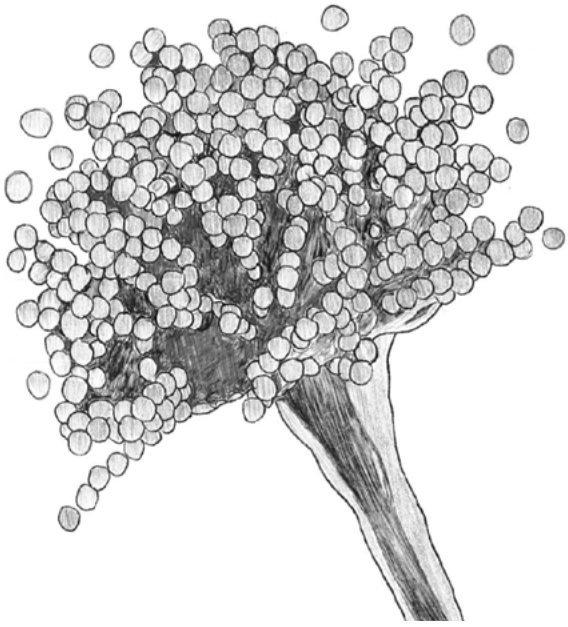
Chapter 14

Summary and conclusion

English
Nederlands



Summary

The immune system plays a key role during infections with *Aspergillus fumigatus*, and can be beneficial as well as detrimental for the host. A compromised status of the immune system or a hypersensitive immune system can make patients susceptible for invasive or allergic aspergillosis respectively. An efficient clearance of the fungus by the immune system is crucial for preventing and inhibiting fungal outgrowth in invasive aspergillosis (IA). However, the host response can also induce damaging immunopathology that can contribute to the pathogenesis of allergic bronchopulmonary aspergillosis (ABPA) and chronic pulmonary aspergillosis (CPA). Within this thesis several studies are described that shed new light on the pathways that regulate the recognition of *A. fumigatus* and on the cytokine networks that govern antifungal host defence against *A. fumigatus*.

Pattern recognition receptors in the host defence against *Aspergillus fumigatus*

Recognition of conserved pathogen associated molecular patterns (PAMPs) by the pattern recognition receptors (PRRs) is the first step in mounting an immune response against a pathogen. Recognition of PAMPs on the *Aspergillus* cell wall is crucial for inducing host defence mechanisms. In **chapter 2** we present an overview of the current knowledge on the PRRs that are involved in initiating host defence mechanisms in response to *Aspergillus* infection. We describe that nearly all classes of PRRs, except RIG-I helicases, are involved in recognition of PAMPs on the *A. fumigatus* cell wall. In addition, we reviewed the role of PRRs in activation of specific immune responses such as neutrophil recruitment, opsonisation, phagocytosis, killing, NETtosis and induction of adaptive immune responses. Although this review illustrates the broad knowledge on PRR signalling in host defence in aspergillosis, many of the PAMPs that activate these receptors remain unknown, and for many of these receptors it remains to be determined how they regulate antifungal immune mechanisms.

One of the mechanisms induced by PRRs is phagocytosis. In immune-competent individuals, phagocytic cells efficiently eliminate *A. fumigatus* spores, thus preventing germination and invasive fungal growth. However, little is known on the molecular mechanisms regulating intracellular killing of *A. fumigatus* by human phagocytes. In **chapter 3**, we describe that swelling of conidia during *A. fumigatus* infection within human monocytes triggers an early and selective induction of the autophagy pathway. It was previously described that conidial swelling results in exposure of PAMPs [1], including β-1,3-glucan [2]. We found that the formation of LC3 positive (auto)phagosomes containing *A. fumigatus* germinating spores was also tightly regulated by β-glucan surface exposure following conidial swelling and germination. Dectin-1 is the pattern recognition receptor on monocytes that recognizes β-glucan, and was previously found to be important for effective antifungal in host defence [3-8]. By using specific inhibitors and cells of patients that are deficient for dectin-1 we were able to show its role in mediating LC3-associated phagocytosis induced by β-glucan. In addition, immunosuppressive therapy with high doses corticosteroids is a major risk factor for the development of aspergillosis [9]. Within this chapter we additionally demonstrated that corticosteroids can interfere with *Aspergillus*-induced LC3-associated phagocytosis by selectively

inhibiting the dectin-1/ spleen tyrosine kinase (Syk) pathway needed for efficient LC3-associated phagocytosis.

Although PRRs initially regulate innate immune cell activation and cytokine release, they create a cytokine environment that regulates the induction of adaptive immune responses such as T-helper cell polarization. Little is known about the T-helper responses induced by *Aspergillus* in humans, and in particular the IL-22 response induced by these cells. Moreover, the IL-22 response to *Aspergillus* recently gained interest because of its protective role in a murine model of invasive aspergillosis [6], and its detrimental role in a model for ABPA [10]. In **chapter 4**, we elucidated the PRR and cytokine pathways that regulate the induction of human *Aspergillus*-induced T-helper responses. We found that IL-17 and IL-22 induction was inhibited by blockade of TLR4 and complement receptor 3, while TLR2 blockade boosted these responses, revealing a potential inhibitory role for TLR2. IFNγ induction in this study was only influenced by blockade of complement receptor 3. In contrast to previous reports [3,6,10], we found that dectin-1 blockade did not affect human *Aspergillus*-induced T-helper responses, and we were able to confirm this with cells isolated from dectin-1 deficient patients. In addition to the PRRs regulating the induction of these T-helper cytokines we demonstrated that the IL-17, IL-22 and IFNγ responses induced by *A. fumigatus* in human PBMCs were almost exclusively T-cell dependent. Furthermore, we characterized the human T-cell subsets that produce IL-22, IL-17, and IFNγ induced by *A. fumigatus*. Of specific interest, we found that the production of IL-22 is not restricted to a specific T-helper subset but is co-expressed in IL-17, and IFNγ positive cells. We further characterized *Aspergillus*-induced IL-22$^+$ CD4$^+$ T-cells and showed that these cells co-express TNFα, a hallmark of the recently described Th22 subset [11]. Moreover, we demonstrate that the induction of this subset is dependent on both IL-1 and TNFα. These last observations point to a role for Th22 cells in the *Aspergillus*-induced T-cell responses which would be interesting to investigate because of their described beneficial role in invasive aspergillosis [6] and detrimental role in ABPA [10].

Many patients with severe asthma demonstrate sensitization to fungal allergens. The allergic phenotype in these patients is primarily caused by the Th2 response which also plays an important role in the progression of fungal sensitization to the more severe form of fungal asthma namely ABPA[12,13]. Moreover the Th2 pathway plays an important role in dampening the protective Th1 responses during invasive aspergillosis [14,15], thereby impairing fungal clearance and decreasing survival. In **chapter 5** we explored the PRRs that regulate the *Aspergillus*-induced Th2 response. We found that *A. fumigatus* conidia were in particular capable of inducing Th2 cytokine responses whereas hyphae and other pathogens such as *Candida albicans* and *Staphylococcus aureus* induced IL-5 and IL-13 to a lesser extent. Similar to the findings in the previous chapter we found that CR3 plays an important role in the induction of Th2 responses while TLR2, TLR4 and dectin-1 neutralization did not significantly influence the Th2 response. Interestingly, we were able to demonstrate that, compared to non-allergic controls, ABPA patients have a significantly increased Th2/Th1 response ratio when their PBMCs are exposed to *Aspergillus ex vivo*. Since it is thought that a distorted balance between Th2 and Th1 responses is responsible for the detrimental immunopathology and impaired fungal clearance in ABPA, we attempted to reverse this ratio by exploring immunomodulatory drugs

in vitro. Neutralization of IL-1 signalling by anakinra (recombinant IL-1 receptor antagonist) or TNFα by soluble TNF receptor resulted in decreased Th2 responses, but unfortunately also decreased Th1 responses. However, recombinant IFNγ (immukine) that is used in clinical practice could dampen *Aspergillus*-induced Th2 responses, while leaving Th1 responses unaffected, and even supplement IFNγ deficiency.

The NACHT-LRR receptors (NLRs) NOD1 and NOD2 are intracellular pattern recognition receptors that play a major role in the recognition of bacterial peptidoglycan derivates by innate immune cells [16-20]. Unexpectedly, accumulating evidence indicated a role for NOD2 in the host response against *A. fumigatus* [21,22]. In **chapter 6** we investigated the role of NOD1 and NOD2 in the immune response to *Aspergillus* and susceptibility to invasive aspergillosis. By using cells of patients with Crohn's disease, that carry a NOD2 mutation, we observed that NOD2 is required for mounting an efficient innate and T-cell cytokine response against *A. fumigatus*. We additionally explored the role of NOD1 and NOD2 in susceptibility to invasive aspergillosis in an *in vivo* mouse model, and observed that these receptors differentially regulate the host response against *A. fumigatus*. Both NOD1 and NOD2 deficiency protects immunosuppressed mice during invasive aspergillosis. NOD2 deficient mice develop inflammation and severe symptoms, but survive the infection, whereas NOD1 deficient mice very rapidly clear the infection. *In vitro* experiments with splenocytes demonstrate that NOD1 deficient cells are more efficient than wild-type and NOD2 deficient cells in generating a cytokine response against *Aspergillus*. In addition we demonstrate that bone marrow derived macrophages from NOD1 deficient mice are more efficient in generating reactive oxygen species, which was associated with an increased fungal killing capacity compared to wild-type mice. Collectively, the data in this chapter demonstrates that NOD1 has a detrimental inhibitory effect on the antifungal host defence against *A. fumigatus*.

Cytokine networks in the host defence against *Aspergillus fumigatus*

Intercellular communication is crucial for mounting an effective immune response, for example for effective communication between the innate and adaptive immune system. In particular the cytokine Interleukin-1 has a prominent role in the immune response. It plays a crucial role in regulating the recruitment of neutrophils, either directly or through inducing T-helper responses [23,24]. In **chapter 7** an overview of the literature is presented on the role of the IL-1 cytokine family and in particular the classical members of the IL-1 family, IL-1β, and IL-1α, and their role in the host defence against *A. fumigatus*.

A. fumigatus employs various strategies to evade immune recognition. Rodlets and melanin, that are present on the conidial surface, shield PAMPs that elicit pro-inflammatory host responses [1,25,26]. Galactosaminogalactan (GAG), a cell wall component of *A. fumigatus* can act as a virulence factor [27]. This polysaccharide that is shed into the host environment during *Aspergillus* vegetative growth induces immunosuppressive effects and diminishes neutrophil recruitment favouring *A. fumigatus* infection in mice [27]. However, the mechanism through which GAG induces immunosuppressive effects as well as its effects on the human immune response were unknown.

In **chapter 8** we demonstrate that GAG is not only immunosuppressive in mice, but can also inhibit human pro-inflammatory T-helper responses such as Th17 responses in human PBMCs. We identified that the potent anti-inflammatory properties of GAG were dependent on the capacity of GAG to induce interleukin-1 receptor antagonist (IL-1Ra), a potent anti-inflammatory cytokine that blocks IL-1 signalling. In addition, we observed that IL-1Ra deficient mice are protected against the immunosuppresive effects of GAG in invasive pulmonary aspergillosis. Moreover, the observation that GAG similar to IL-1Ra can protect mice from DSS-induced colitis identifies GAG or a derivative structure of GAG as a potential therapy for IL-1-mediated diseases such as joint, bone and muscle diseases and even very common inflammatory diseases, such as diabetes and gout.

Due to the important role of Interleukin-1 in host defence and in particular the host defence against *Aspergillus*, it can be anticipated that common genetic variations that influence IL-1 activity can have impact on the susceptibility to and the outcome of invasive aspergillosis. In **chapter 9** we describe that common genetic variations in *IL1B* and *IL1RN* result in altered *Aspergillus*-induced cytokine signalling in PBMCs isolated from healthy volunteers. Moreover, in a cohort of solid organ transplant patients these genetic variations and a polymorphism in the *BDEF1* gene encoding for β-defensin, a potent antimicrobial peptide, were highly associated with fungal colonization and invasive aspergillosis. This association highlights the importance of the IL-1 axis and antimicrobial peptides in the defence against fungi in transplant patients.

The IL-1 family does not only consist of IL-1α, IL-1β, and IL-1Ra, but also includes other members such as IL-18 that regulates Th1 responses and IL-33 that regulates Th2 responses. Some members have recently been renamed IL-36α (IL-1F6), IL-36β (IL-1F8), IL-36γ (IL-1F9) and IL-36Ra (IL-1F5) due to new insight in their biological function [28]. These IL-36 cytokines elicit their effects through the IL-36 receptor (IL-36R) pathway and received a lot of interest recently, since they have been found to play a major role in the pathogenesis of psoriasis [29-31]. In **chapter 10** we present a general overview of the current knowledge on the IL-36 cytokine family by reviewing the biology of the IL-36 cytokines and their role in disease. This review describes the discovery of the new members of the IL-1 family, followed by the known biological functions of the IL-36 cytokines. The processing and secretion of the IL-36 antagonists and agonists, and the general role of IL-36 signalling in the immune response is described. Furthermore, we discuss the literature on the role of IL-36 in a wide variety of inflammatory diseases, and give future perspectives about research on the role of IL-36 cytokines.

Although the IL-36 cytokines seem to play a prominent role in the skin they have also been found to be important for the recruitment of neutrophils to the airways [32]. The IL-36R is primarily expressed on naive CD4 T-cells, and the IL-36R pathway is involved in the activation of T-cells [33]. For these reaseons we investigated, in **chapter 11**, the role for the IL-36R pathway in the host response against *Aspergillus*. We found that *Aspergillus* induced expression of both the IL-36 cytokines and their antagonist IL-36Ra, and that the IL-36 receptor pathway plays an important role in the regulation of Th1 and Th17 responses. However, inhibition of the IL-36 pathway did not affect *Aspergillus*-induced Th2 and regulatory T-cell responses. We provided additional evidence for the biological importance

of the IL-36 pathway by blocking endogenous IL-36Ra, which resulted in the increase of *Aspergillus*-induced IL-17 and IFNγ responses. Furthermore, in this chapter we also decipher for the first time the innate recognition pathways, namely TLR4 and dectin-1/Syk, which are involved in the induction of IL-36γ by *A. fumigatus.*

In **chapter 12** we present a case series consisting of patients included in a clinical trial who received adjunctive immunotherapy with recombinant IFNγ and several patients for whom the treatment was given as "last resort therapy". The data in this chapter provides proof-of principle that IFNγ is capable of increasing the capacity of PBMCs from these patients to produce higher levels of pro-inflammatory cytokines upon stimulation *ex vivo*. These data support the rationale for IFNγ treatment in patients with severe invasive fungal infections that have a decreased inefficient immune response, and provide an important argument that further studies including larger numbers of patients are warranted to confirm the clinical usefulness of (IFNγ) adjuvant immunotherapy in invasive fungal infection.

General conclusion

The fact that aspergillosis is rarely found in individuals with an unaffected immune system tells us that the human immune system has adequately evolved to prevent aspergillosis. Meaning that in patients with a suppressed immune system key components of the host response that prevent infection with *Aspergillus* are neutralized. By investigating how *Aspergillus* is recognised by the host and which cytokine signalling networks that regulate immune responses to *Aspergillus fumigatus*, we can identify these key components that, if defective or working suboptimal, render patients more susceptible to aspergillosis. However, the inflammatory response that is induced to counter *Aspergillus* infection can become detrimental or skewed towards an allergic phenotype, which is an important aspect in the pathogenesis of fungal asthma. These two faces of the host response against *Aspergillus* are important to keep in mind when identifying eligible targets for the development of novel immunomodulatory therapies that can be given in combination with currently available antimycotic therapy.

Samenvatting

Het immuunsysteem speelt een fundamentele rol tijdens infecties met de schimmel *Aspergillus fumigatus,* en heeft zowel heilzame als nadelige gevolgen voor de gastheer. Patiënten met een gecompromitteerde immuun status zijn gevoelig voor het ontwikkelen van aspergillose, en individuen die een overgevoelige afweerrespons tegen *Aspergillus* opwekken lopen risico om een allergische aspergillose te ontwikkelen. Het efficiënt opruimen van ingeademde schimmelsporen door het immuunsysteem is cruciaal voor het voorkomen van uitgroei van schimmels in de longen. Het immuunsysteem kan echter ook nevenschade veroorzaken in de vorm van immunopathologie, hetgeen bijdraagt aan de ontwikkeling van allergische bronchopulmonale aspergillose (ABPA) en chronische pulmonale aspergillose (CPA). In dit proefschrift worden verschillende studies beschreven die nieuw licht werpen op hoe het immuunsysteem de schimmel *A. fumigatus* herkent en welke cytokine netwerken een rol spelen in de afweer tegen *A. fumigatus.*

Pathogeen herkenning receptoren in de afweer tegen *Aspergillus fumigatus*

De herkenning van pathogeen specifiek moleculaire patronen (PAMPs) door gespecialiseerde pathogeen herkenningsreceptoren (PRRs) op afweercellen, is de eerste stap in de afweer tegen een binnengedrongen pathogeen. Herkenning van PAMPs op de celwand van *Aspergillus* is cruciaal voor het activeren van afweermechanismen. In **hoofdstuk 2** wordt een overzicht gepresenteerd van de huidige kennis op het gebied van herkenning van *Aspergillus* door het immuunsysteem en wordt beschreven hoe de gespecialiseerde PRRs specifieke afweermechanismen activeren. We constateren dat haast alle bekende PRRs, behalve RIG-I helicase, een rol spelen in de herkenning van PAMPs op de celwand van *A. fumigatus*. Daarnaast wordt een overzicht gegeven van de wijze waarop deze immuunreceptoren specifieke afweermechanismen zoals: fagocytose, cytokine signalen en de adaptieve afweerrespons activeren. Alhoewel in het overzicht de brede kennis op het gebied van herkenning van *Aspergillus* in de afweerreactie wordt beschreven is het onbekend welke moleculaire patronen op de celwand van *Aspergillus* herkend worden, en bij veel immuunreceptoren is het onbekend hoe zij de antischimmel afweerreactie in gang zetten.

Fagocytose is één van de belangrijkste mechanismen die door PRRs gereguleerd wordt. In individuen met een goed functionerend immuunsysteem ruimen de fagocyterende immuuncellen de ingeademde schimmelsporen efficiënt op. Tot op heden is weinig bekend over de regulerende mechanismen in afweercellen die de gefagocyteerde *Aspergillus* sporen doden. In **hoofdstuk 3** wordt beschreven dat het ontkiemen (opzwellen) van *Aspergillus* sporen in monocyten resulteert in een selectieve inductie van autofagie. Het opzwellen van sporen leidt tot blootstelling van PAMPs, zoals β-1,3-glucan [2], die door de PRRs op afweercellen [1] herkend kunnen worden. We vonden dat de vorming van LC3 positieve (auto)fagosomen, met daarin ontkiemende *A. fumigatus* sporen, gereguleerd worden door de oppervlakte expressie van β-glucan. Dectine-1 is de receptor op monocyten die β-glucan herkent, en van deze receptor is eerder beschreven dat deze cruciaal is voor een doeltreffende afweer tegen schimmels [3-8]. Met behulp van specifieke dectine-1 remmers en cellen van patiënten, die deficiënt zijn voor dectine-1, hebben we aangetoond dat herkenning

van β-glucan door dectine-1 van fagocytose met associatie van LC3 reguleert. Immunosuppressieve behandeling met hoge doseringen van corticosteroïden is een belangrijke risicofactor voor de ontwikkeling van aspergillose [9]. In dit hoofdstuk hebben we aangetoond dat corticosteroïden interfereren met LC3 geassocieerde fagocytose van *Aspergillus* door selectief de herkenning van β-glucan door dectine-1 te remmen. Men zou daarom kunnen aannemen dat corticosteroïden bijdragen aan de gevoeligheid voor aspergillose doordat de LC3 fagocytose, die nodig is voor het opruimen van *Aspergillus,* specifiek door corticosteroïden worden geremd.

Hoewel PRRs aanvankelijk cel activatie en cel-cel communicatie door middel van cytokines in het aangeboren immuunsysteem reguleren, zorgen zij er indirect voor dat er een cytokine milieu gecreëerd wordt dat de inductie van adaptieve immuunrespons reguleert. Er is nog weinig bekend over de adaptieve T-helper immuunrespons tegen *Aspergillus* in mensen, en in het bijzonder de Interleukine(IL)-22 respons door T-helper cellen. Deze IL-22 respons is recentelijk onder de aandacht gekomen doordat verschillende studies hebben aangetoond dat deze cytokine respons belangrijk is voor de bescherming van de gastheer tegen invasieve aspergillose [6]. Deze cytokine kan echter een schadelijke rol spelen in allergische aspergillose [10]. In **hoofdstuk 4** hebben we onderzocht hoe PRRs en verschillende cytokines van het aangeboren immuunsysteem de inductie van de T-helper respons tegen *Aspergillus* reguleren. We vonden enerzijds dat inductie van de T-helper cytokines IL-17 en IL-22 geremd werd door blokkade van Toll-like receptor (TLR)4 en complement receptor (CR)3, terwijl anderzijds blokkade van TLR2 deze reacties versterkte. De inductie van de T-helper cytokine Interferon(IFN)γ werd alleen beïnvloed door blokkade van complement receptor 3. In tegenstelling tot eerdere studies die uitgevoerd zijn in muizen [3,6,10], vonden we met behulp van dectine-1 blokkade, dat dectine-1 slechts een beperkte rol speelt in de humane T-helper response geïnduceerd door *Aspergillus.* We konden deze bevinding bevestigen met cellen afkomstig van dectine-1 deficiënte patiënten. Naast de regulatie van de T – helper respons door pathogeen herkenningsreceptoren hebben we aangetoond dat de IL-17, IL-22 en IFNγ respons op *Aspergillus* uitsluitend T-cel afhankelijk is. Vervolgens hebben we de T-cel subsets die deze cytokines produceren verder gekarakteriseerd. We vonden in het bijzonder dat de productie van de cytokine IL-22 niet beperkt is tot een specifieke T-helper subset, maar geproduceerd kan worden door T-cellen die ook IL-17 en IFNγ tot expressie brengen. De subset T-cellen die uitsluitend IL-22 produceren hebben we verder gekarakteriseerd, en we vonden dat deze cellen naast IL-22 ook tumour necrose factor(TNF) α tot expressie brengen, een kenmerk van de recent beschreven Th22 subset [11]. Verder hebben we aangetoond dat de inductie van deze subgroep afhankelijk is van zowel IL-1 en TNFα . Deze data wijzen op een rol voor de recentelijk beschreven Th22 cellen in de afweer tegen *Aspergillus*, wat interessant is om verder te onderzoeken door de eerder beschreven gunstige effecten van IL-22 in invasieve aspergillose [6] en de nadelige effecten in allergische aspergillose [10].

Patiënten met ernstige vormen van astma hebben vaak positief testresultaat tegen allergenen afkomstig van schimmels. De allergische reactie wordt in deze patiënten gedreven door de T-helper 2 respons, die ook een belangrijke rol speelt in de progressie van overgevoeligheid voor schimmelallergenen als ziektebeeld van allergische bronchopulmonale aspergillose (ABPA)

[12,13]. Daarnaast is de Th2 respons in staat om de protectieve Th1 response tegen *Aspergillus* te onderdrukken [14,15], waardoor het uitgroeien van *Aspergillus* sporen minder goed onderdrukt wordt, hetgeen uiteindelijk leidt tot een verhoogde schade in de longen en een hogere mortaliteit. In **hoofdstuk 5** hebben we onderzocht welke PRRs de inductie van de Th2 respons door *Aspergillus* reguleren. We vonden dat uitsluitend de sporen van *Aspergillus* een allergische Th2 respons opwekken, terwijl hyfen van *Aspergillus* en andere pathogenen de Th2 respons niet sterk induceren. Zoals in het vorige hoofdstuk beschreven vonden we dat CR3 wederom een belangrijke rol speelt in de inductie van de Th2 response door *Aspergillus*. We constateerden dat de receptoren TLR2, TLR4 en dectine-1 geen rol speelden in de inductie van de Th2 respons door *Aspergillus*. Een interessante observatie in deze studie was dat cellen van ABPA patiënten een verhoogde Th2/Th1 ratio hebben, wanneer de respons wordt vergeleken met niet allergische individuen. In de wetenschap wordt er van uitgegaan dat een verstoorde Th2/Th1 ratio ten grondslag ligt aan de schadelijke immunopathologie in ABPA. Derhalve hebben we geprobeerd de verstoorde Th2/Th1 ratio met immunomodulatoire medicijnen te herstellen. Neutralisatie van IL-1 door anakinra (recombinant IL-1 receptor antagonist) of TNFα door etanercept (recombinant TNFα receptor II) resulteerde in een verlaging van de Th2 respons op *Aspergillus*, maar neutraliseerde ook de inductie van de Th1 respons. Recombinant IFNγ (immukine) werd gebruikt om de Th2 respons te neutraliseren en interfereerde daarbij niet met de Th1 respons. Deze observaties laten zien dat behandeling met recombinant IFNγ een mogelijke immunomodulatoire therapie is voor ABPA door het remmen van de Th2 as en het ondersteunen van de Th1 respons tegen *Aspergillus*.

De NACHT-LRR receptoren (NLRs) NOD1 en NOD2 zijn intracellulaire PRRs die een cruciale rol spelen in de herkenning van bacterieel peptidoglycaan door cellen van het aangeboren immuunsysteem [16-22]. Tegen verwachting in is er bewijs dat de NOD2 receptor mogelijk een rol speelt in de afweer tegen *A. fumigatus* [21,22]. In **hoofdstuk 6** hebben we de rol van NOD1 en NOD2 onderzocht in de afweer tegen *Aspergillus* en de gevoeligheid voor het ontwikkelen van aspergillose. Door cellen te gebruiken van patiënten met de ziekte van Crohn, bij wie de NOD2 receptor niet functioneel is, hebben we aangetoond dat NOD2 nodig is voor het opwekken van een immuun respons tegen *Aspergillus* door zowel het aangeboren immuunsysteem als het adaptieve immuunsysteem. Daarnaast hebben we de rol van NOD1 en NOD2 onderzocht in een muismodel voor invasieve aspergillose. We vonden dat muizen die deficiënt zijn in ofwel NOD1 dan wel NOD2 beschermd zijn tegen een dodelijke infectie met *Aspergillus*. NOD2 deficiënte muizen ontwikkelden echter nog ernstige symptomen van de ziekte. Aan de andere kant lieten de NOD1 deficiënte muizen weinig symptomen van de infectie zien en zij herstelden zich snel na de infectie. Door cellen van deze muizen *in vitro* te gebruiken hebben we aangetoond dat NOD1 deficiënte cellen efficiënter zijn in het opwekken van een cytokinerespons tegen *Aspergillus* dan cellen van wild-type of NOD2 deficiënte muizen. Daarnaast hebben we aangetoond dat macrofagen die NOD1 missen beter in staat zijn een oxidatieve burst te genereren, en daarbij beter in staat zijn om sporen van *Aspergillus* te doden. De resultaten in dit hoofdstuk tonen aan dat NOD1 een slechte invloed heeft op de afweer tegen *Aspergillus*, omdat deficiëntie van NOD1 geassocieerd wordt met bescherming tegen

dodelijke *Aspergillus* infectie en een verhoogde capaciteit om een effectieve immuunrespons tegen de schimmel op te wekken. Om deze redenen wordt gesteld dat NOD1 mogelijk een toekomstig doelwit is voor nieuwe immunotherapie om de afweer tegen *Aspergillus* te verbeteren gedurende een periode waarin het immuunsysteem gecompromitteerd is.

Cytokine netwerken in de afweer tegen *Aspergillus fumigatus*

De mate waarin verschillende immuuncellen met elkaar communiceren is essentieel voor het opwekken van een efficiënte immuunrespons. Een effectieve communicatie tussen het aangeboren afweersysteem en het adaptieve immuunsysteem door cytokines speelt een belangrijke rol in het polariseren van de adaptieve immuunrespons. Wanneer dit echter niet efficiënt gebeurt kan de adaptieve afweerreactie de aangeboren afweer niet versterken. De cytokine IL-1 speelt een prominente rol in de immuunrespons. Deze cytokine speelt een cruciale rol in het rekruteren van neutrofiele granulocyten, zowel direct, alsmede door indirect eerst T-helper responsen te induceren die vervolgens granulocyten rekrutering activeren [23,24]. In **hoofdstuk 7** focust de samenvatting van de literatuurstudie op de rol van de IL-1 cytokine familie in de afweer tegen *A. fumigatus*, en met name de klassieke leden van de IL-1 familie: IL-1β en IL-1α. Ook wordt de potentie van de IL-1 pathway als een potentieel doelwit voor immunotherapie in aspergillose geëvalueerd. IL-1 is een zeer potente activator van de proinflammatoire immuun respons. Juist om deze reden zou het blokkeren van L-1 gebruikt kunnen worden om *Aspergillus* geassocieerde immunopathologie te voorkomen die beschreven is in de ziektebeelden ABPA, CPA en chronische granulomateuze ziekte (CGD). Echter het versterken van de IL-1 as zou op zijn beurt een potentiële therapie kunnen zijn voor immuungecompromitteerde patiënten, die een verbetering van schimmel klaring kan bewerkstelligen. In dit hoofdstuk is ook ingegaan op de wijze waarop polymorfismen in genen van de IL-1 pathway genetische markers zijn om gevoeligheid voor aspergillose te voorspellen en risicopatiënten te identificeren die baat kunnen hebben bij intensieve zorg en profylactische antimycotische therapie.

A. fumigatus gebruikt verschillende strategieën waardoor het voorkomt door het immuunsysteem herkend te worden. Rodlets en melanine zijn aanwezig op het oppervlak van de sporen en schermen PAMPs af die de immuunrespons kunnen activeren [1,25,26]. Galactosaminogalactan (GAG), een molecuul in de celwand van *A. fumigatus,* kan zich voordoen als een virulentie factor [27]. Deze suiker wordt uitgescheiden tijdens de groei van *Aspergillus* en remt het rekruteren van neutrofiele granulocyten, hetgeen ten gunste van de schimmel is [27]. Het was onbekend hoe GAG deze immuun onderdrukkende effecten induceerde, daarom hebben we deze effecten in **hoofdstuk 8** nader onderzocht. In dit hoofdstuk wordt bewezen dat GAG het menselijk immuunsysteem onderdrukt. Deze polysacharide is in staat om de Th17 respons in humane immuuncellen te remmen. De immunosuppressieve effecten van GAG zijn onderzocht en aangetoond is dat GAG IL-1 receptor antagonist (IL-1Ra) een potente anti-inflammatoire cytokine induceert. Daarnaast hebben we geconstateerd dat muizen die voor IL-1Ra deficiënt zijn beschermd tegen de immunosuppressieve effecten van GAG. Dit creëert mogelijkheden voor nieuwe behandelingen tegen acute invasieve aspergillose gericht op IL-1Ra. Ook hebben we geconstateerd dat therapie met GAG muizen

beschermt tegen inflammatoire ziekten zoals colitis ulcerosa en allergische aspergillose. Deze observatie uit het onderzoek kan er toe leiden dat GAG, of een afgeleide structuur van GAG, gebruikt zou kunnen worden voor behandeling van IL-1 gedreven inflammatoire ziekten zoals colitis, gewricht- en spierziekten, diabetes en jicht.

Door de belangrijke rol van IL-1 in de afweer, en in het bijzonder de afweer tegen *Aspergillus*, is het aannemelijk dat genetische variaties die de activiteit van IL-1 beïnvloeden een rol spelen in de gevoeligheid voor aspergillose en het klinisch verloop van aspergillose. In **hoofdstuk 9** beschrijven we dat veel voorkomende genetische variaties in *IL1B* en *IL1RN* (de genen voor IL-1β en IL-1Ra) de cytokine respons tegen *Aspergillus* door afweercellen van gezonde vrijwilligers beïnvloeden. Ook is in een groep patiënten die orgaantransplantatie ondergingen deze genetische variaties, en een variatie in het *DEFB1* gen dat codeert voor de potente antimicrobiele peptide β-defensin, geassocieerd met een verhoogde gevoeligheid voor invasieve schimmelinfecties. Deze associatie benadrukt hoe belangrijk de IL-1 as en β-defensin zijn in de afweer tegen schimmelinfecties bij patiënten met een orgaantransplantatie. De onderzochte genetische variaties kunnen in de toekomst gebruikt worden om risicoanalyses te maken. Hierdoor krijgen transplantatiepatiënten een gepersonaliseerde profylactische antimycotische therapie en worden zij intensiever diagnostisch gescreend.

De IL-1 cytokine familie bestaat niet alleen uit: IL-1α, IL-1β, en IL-1Ra, maar ook uit andere leden zoals IL-18 dat de Th1 respons reguleert en uit IL-33 dat op zijn beurt de Th2 respons reguleert. Sommige IL-1 familieleden zijn door nieuwe inzichten in hun biologische functie[28] recentelijk herbenoemd, namelijk: IL-36α (IL-1F6), IL-36β (IL-1F8), IL-36γ (IL-1F9) en IL-36Ra (IL-1F5). De IL-36 cytokines oefenen hun biologische functie uit door de IL-36 receptor te binden en te activeren, en krijgen de laatste tijd steeds meer aandacht vanwege hun rol in de pathogenese van psoriasis [29-31]. In **hoofdstuk 10** wordt een overzicht gegeven van de huidige kennis over de IL-36 cytokine familie, met een focus op de biologie van deze cytokines in verschillende ziektes. Dit overzichtsartikel beschrijft hoe de nieuwe IL-1 familieleden ontdekt zijn en beschrijft de biologische functies van deze cytokines. Ook is in dit hoofdstuk beschreven hoe de IL-36 agonisten en antagonisten geproduceerd worden, en hun algemene rol in het immuunsysteem. Tenslotte wordt op basis van de wetenschappelijke literatuur de rol van de IL-36 cyokines in een breed spectrum van inflammatoire ziektes beschreven en wordt gespeculeerd over de mogelijke toekomstperspectieven van IL-36 cytokines voor de medische wetenschap.

Naast het feit dat de IL-36 cytokines een belangrijke rol spelen in homeostase van de huid, zijn deze cytokines ook belangrijke signaalmoleculen voor het rekruteren van granulocyten naar de luchtwegen [32]. De IL-36 receptor zit op het oppervlak van naïeve CD4 T-cellen en speelt een belangrijke rol in de activatie van deze cellen [33]. Derhalve is in **hoofdstuk 11** de rol van IL-36 in de afweer tegen *Aspergillus* onderzocht. We vonden dat *Aspergillus* mRNA expressie van de IL-36 cytokines en hun antagonist IL-36Ra kon induceren. Daarnaast vonden we dat de IL-36 cytokines de inductie van de Th1 en Th17 respons door *Aspergillus* reguleerden. Deze regulatie is vervolgens bevestigd door endogeen IL-36Ra met antilichamen weg te vangen wat resulteerde in een

versterking van de Th1 en Th17 inductie door *Aspergillus*. Verder is in het onderzoek aangetoond dat de PRRs TLR4 en dectine-1 de inductie van IL-36γ door *Aspergillus* reguleren. De bevinding dat een nieuwe cytokine familie de Th1 en Th17 inductie door *Aspergillus* reguleert, zorgt voor nieuwe mogelijke doelen voor de ontwikkeling van een immunotherapie voor aspergillose. De screening van genetische variaties in deze cytokines zou kunnen helpen om een verhoogde gevoeligheid voor aspergillose te voorspellen.

Door vaker voorkomende resistentie van *A. fumigatus* voor anti-mycotica is er een vraag naar alternatieve behandelingsstrategieën. Modulatie van de afweerreactie wordt geprezen als een veelbelovende therapie die in combinatie met de standaardtherapie de mortaliteit van invasieve schimmelinfecties kan terugbrengen. In **hoofdstuk 12** worden casussen beschreven van patiënten met invasieve candidiasis. De patiënten zijn geïncludeerd in een klinische trial en ontvingen naast de standaard zorg additionele immunotherapie behandeld met recombinant IFNγ. Ook zijn verschillende patiënten beschreven met invasieve schimmelinfecties die als "laatste redmiddel" immunotherapie met IFNγ kregen. De resultaten van dit onderzoek geven een eerste bewijs dat immunotherapie met IFNγ de capaciteit van immuuncellen om pro-inflammatoire cytokines te produceren kan versterken. Deze data ondersteunen de gedachte dat patiënten met ernstige invasieve schimmelinfecties baat zouden hebben bij immunotherapie met IFNγ. Daarnaast vormen ze de basis voor het ontwikkelen van nieuwe studies met grotere groepen patiënten om klinische toepassing van immunotherapie (met INFγ) in invasieve schimmelinfecties diepgaand te onderzoeken.

Conclusies en toekomstperspectieven

Het feit dat aspergillose zelden voor komt in individuen met een intact immuunsysteem zegt ons dat het menselijk immuunsysteem adequaat is geëvolueerd om infecties met *Aspergillus* te voorkomen. Hetgeen betekend dat in patiënten met een gecompromitteerd imuunsysteem bepaalde immunologische herkennings- en signaalmechanismen, die een rol spelen in de afweer tegen *Aspergillus,* niet goed functioneren. Door de wetenschappelijke kennis van deze mechanismen toe te passen worden cruciale componenten geïdentificeerd die, indien defect, patiënten gevoelig maken om aspergillose te ontwikkelen. De immuunrespons die geïnduceerd wordt om *Aspergillus* te klaren kan echter ook nadelige gevolgen hebben door zich bijvoorbeeld te ontwikkelen tot een allergisch fenotype of schade te berokkenen aan de gastheer. Deze twee keerzijden van de afweer tegen *Aspergillus* zijn belangrijk om in gedachten te houden bij het identificeren van potentiële nieuwe targets voor immunotherapie die gebruikt zouden kunnen worden in combinatie met de huidig beschikbare anti-mycotische therapieën.

Het onderzoek in dit proefschrift wil een bijdrage leveren aan het snel ontwikkelende onderzoeksveld dat de afweer tegen *Aspergillus* onderzoekt. Door de pathogeen herkennings- en cytokine signaalwegen te onderzoeken die de afweerreactie tegen *Aspergillus* reguleren worden in dit proefschrift nieuwe perspectieven gethematiseerd van de verschillende aspecten die de complexe interactie tussen *Aspergillus* en het immuunsysteem van de mens beschrijven.

De studies in dit proefschrift hebben bijgedragen aan de optimalisatie van onze kennis van deze interacties. Echter de huidige kennis van de interactie tussen *Aspergillus* en gastheer is verre van compleet en veel wetenschappelijke uitdagingen vragen om verder onderzoek. Toekomstige studies moeten uitwijzen welke PAMPs van *Aspergillus* de PRRs CR3, TLR2, TLR4, NOD1, en NOD2 activeren. Ook is onbekend welke afweerreceptoren de celwandmoleculen chitine en galactosaminogalactan herkennen. Voor immunomodulatoire therapieën is het moeilijk te concluderen of de proinflamatoire afweerreactie versterkt moet worden om efficiëntere klaring van de infectie te krijgen, dan wel moet worden afgeremd om schadelijke immunopathologie te voorkomen. Om de complexiteit van het immuunsysteem te ontrafelen en te begrijpen moet het immuunsysteem *Aspergillus* verder onderzocht worden zodat de eerder geformuleerde vragen in de toekomst beantwoord worden. Tenslotte zal het toekomstig onderzoek naar nieuwe cytokine en herkenningsmechanismen ons helpen om voor patiënten nieuwe therapieën en screening van risicomarkers voor aspergillose te ontwikkelen.

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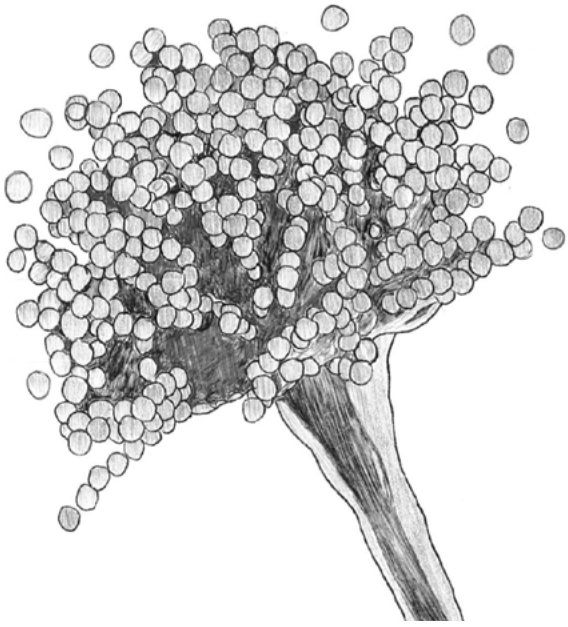
Chapter 15

Acknowledgements

List of publications

Curiculum vitae



Na 4 jaar knallen op groot verzet is dit boekje het eindresultaat van mijn promotieonderzoek. Veel werk in een korte tijd verzetten betekent dat de tijd voorbijvliegt, maar kijk met veel plezier terug op deze periode. Dit boekje is tot stand gekomen met directe- en indirecte- bijdrages van unieke personen die mij gesteund hebben. In dit dankwoord wil ik mijn dank uiten aan iedereen die mij heeft bijgestaan bij het volbrengen van mijn promotie en in het bijzonder wil ik onderstaande personen bedanken.

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Pastis = red wine = ti'punch > IL-22 + IL-1 + GM-CSF

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1. van de Veerdonk FL, **Gresnigt MS**, Oosting M, van der Meer JW, Joosten LA, Netea MG, *et al.* Protective host defense against disseminated candidiasis is impaired in mice expressing human interleukin-37. Frontiers in microbiology 2014;5:762.

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Curiculum Vitae

Mark Gresnigt was born in Utrecht on the April 18th 1986. He spent his youth in Houten and moved at age 11 to the small town of Tubbergen, where he attended the St. Canisius Higher General Secondary Education between 1998 and 2000. From 2000 until 2003, he attended the St. Canisius Higher General Secondary Education in Almelo where he followed a technical profile with biology as an elective course. Subsequently, he studied Biology and Medical Laboratory Research in Enschede at the Saxion University of Applied Sciences. During his studies, he specialized in medical microbiology and acquired practical experience during an internship in the St. Antonius hospital in Nieuwegein on the determination of microorganisms from clinical samples. During his studies he gained interest in the fields of, microbiology, immunology and genetic research. Therefore, he performed a research internship in the Gelre hospital in Aperldoorn, where he studied polymorphisms in the genome of *Mycobacterium tuberculosis* that lead to resistance against antibiotics. In 2007 he obtained his Bachelor of Applied Science degree with the designation *Cum Laude*. After his Bachelor studies Mark worked as a laboratory technician for 6 months at the department of Medical Microbiology and Infection prevention of the Gelre Hospital in Apeldoorn, and subsequently continued with his studies at the Radboud University Nijmegen. There he studied Medical Biology and performed a Masters internship in the department of Microbiology of the Radboud University Nijmegen Medical Centre where he constructed transgenic sex-specific green fluorescent protein expressing malaria gametocytes. He performed a second Masters internship in the department of Experimental Internal Medicine on the anti-inflammatory properties of granulocytes. He obtained his Master of Science degree in 2010 with the designation *Cum Laude*. During the holidays of both of his studies he worked as a laboratory assistant at the St. Antonius Hospital and the Gelre Hospital, in both hospitals at the department of Microbiology. After his studies, he stayed at the department of Experimental Internal Medicine, and worked on a PhD project that investigated the pattern recognition and cytokine signalling pathways in the host defence against *Aspergillus fumigatus,* under the mentorship of Frank van de Veerdonk MD. PhD, Prof. Leo Joosten PhD, Prof. Mihai Netea PhD. MD. During his PhD project, Mark presented his results at numerous international scientific conferences and went to the Institute Pasteur in Paris to perform *in vivo* experiments with bioluminescent *Aspergillus* and went to the Aberdeen fungal group to perform live imaging on phagocytosis of *Aspergillus*. He will continue researching the antifungal host defence against opportunistic pathogens such as *Aspergillus* and mucorales, and will visit the research group of Prof. Charles Dinarello in the summer of 2015 to study the biology of the novel cytokine IL-38.

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